

- 1 -

**Treatment Of Cerebrovascular Disease With Neurogenic Locus Notch Homolog
Protein 3 (NOTCH3) Agents**

Reference To Sequence Listing

- 5 This application includes a Sequence Listing submitted electronically as a text file named 18923806502SEQ, created on May 30, 2022, with a size of 364 kilobytes. The Sequence Listing is incorporated herein by reference.

Field

- 10 The present disclosure relates generally to methods of treating subjects having a cerebrovascular disease with Neurogenic Locus Notch Homolog Protein 3 (NOTCH3) agents, and methods of identifying subjects having an increased risk of developing a cerebrovascular disease.

15 **Background**

- Cerebrovascular disease includes any abnormality of the brain resulting from a pathologic process of a blood vessel. A pathologic process of a blood vessel includes any one or more of the following: an occlusion of a blood vessel lumen by thrombus or embolus, a rupture of a blood vessel, an altered permeability of a blood-vessel wall, and increased viscosity or
20 other change in the quality of blood. Cerebrovascular disease is typically readily diagnosable because of how it manifests. Cerebrovascular disease typically manifests as a stroke. A stroke can be characterized as a sudden nonconvulsive, focal neurologic deficit. That is, stroke can be characterized as the death of brain tissue that results from lack of blood flow and insufficient oxygen to the brain. After heart disease and cancer, stroke is the leading cause of death in the
25 United States. In the United States, there are approximately 500,000 cases of stroke annually. And these 500,000 cases give rise to about 175,000 fatalities.

- Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) or CADASIL syndrome, is the most common form of hereditary stroke disorder. The most common clinical manifestations are migraine headaches and
30 transient ischemic attacks or strokes, which usually occur between 40 and 50 years of age, although MRI is able to detect signs of the disease years prior to clinical manifestation of disease. The underlying pathology of CADASIL is progressive hypertrophy of the smooth muscle

- 2 -

cells in blood vessels. No specific treatment for CADASIL is available. While most treatments for CADASIL patients' symptoms – including migraine and stroke – are similar to those without CADASIL, these treatments are almost exclusively empiric, as data regarding their benefit to CADASIL patients is limited.

5 Neurogenic Locus Notch Homolog Protein 3 (NOTCH3) is a receptor for membrane-bound ligands Jagged1, Jagged2, and Delta1 to regulate cell-fate determination. Upon ligand activation through the released notch intracellular domain (NICD), it forms a transcriptional activator complex with RBPJ/RBPSUH and activates genes of the enhancer of split locus. NOTCH3 effects the implementation of differentiation, proliferation and apoptotic programs.

10

Summary

The present disclosure provides methods of treating a subject having a cerebrovascular disease, the methods comprising administering a Neurogenic Locus Notch Homolog Protein 3 (NOTCH3) agent to the subject.

15 The present disclosure also provides methods of treating a subject having a subcortical stroke, the methods comprising administering a NOTCH3 agent to the subject.

The present disclosure also provides methods of treating a subject having an ischemic stroke, the methods comprising administering a NOTCH3 agent to the subject.

20 The present disclosure also provides methods of treating a subject having a hemorrhagic stroke, the methods comprising administering a NOTCH3 agent to the subject.

The present disclosure also provides methods of treating a subject having a parenchymal stroke, the methods comprising administering a NOTCH3 agent to the subject.

25 The present disclosure also provides methods of treating a subject having cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), the methods comprising administering a NOTCH3 agent to the subject.

30 The present disclosure also provides methods of treating a subject with a therapeutic agent that treats or inhibits a cerebrovascular disease, wherein the subject has a cerebrovascular disease, the methods comprising: determining whether the subject has a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide by: obtaining or having obtained a biological sample from the subject; and performing or having performed a sequence analysis on the biological sample to determine if the subject has a genotype comprising the NOTCH3 missense variant nucleic acid molecule

- 3 -

encoding the NOTCH3 predicted gain-of-function polypeptide; and administering or continuing to administer the therapeutic agent that treats or inhibits a cerebrovascular disease in a standard dosage amount to a subject that is NOTCH3 reference, and administering a NOTCH3 agent to the subject; and administering or continuing to administer the therapeutic agent that
5 treats or inhibits a cerebrovascular disease in an amount that is the same as or greater than a standard dosage amount to a subject that is heterozygous or homozygous for the NOTCH3 missense variant nucleic acid molecule, and administering a NOTCH3 agent to the subject; wherein the presence of a genotype having the NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide indicates the subject has an
10 increased risk of developing a cerebrovascular disease.

The present disclosure also provides methods of identifying a subject having an increased risk for developing a cerebrovascular disease, the methods comprising: determining or having determined the presence or absence of a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide in a biological sample
15 obtained from the subject; wherein: when the subject is NOTCH3 reference, then the subject does not have an increased risk for developing the cerebrovascular disease; and when the subject is heterozygous or homozygous for the NOTCH3 missense variant nucleic acid molecule, then the subject has an increased risk for developing the cerebrovascular disease.

The present disclosure also provides therapeutic agents that treat or inhibit a
20 cerebrovascular disease for use in the treatment of a cerebrovascular disease in a subject identified as having: a genomic nucleic acid molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; an mRNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the
25 nucleotide sequence comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; or a cDNA molecule having a
30 nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement

- 4 -

thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof.

The present disclosure also provides NOTCH3 agents for use in the treatment of a
5 cerebrovascular disease in a subject identified as having: a genomic nucleic acid molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; an mRNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a uracil at a
10 position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; or a cDNA molecule having a nucleotide sequence encoding a NOTCH3
15 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof.

20

Brief Description Of The Drawings

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several features of the present disclosure.

Figure 1 shows EXWAS results for the NOTCH3 locus.

25 Figure 2 shows the occurrence of cases and controls in the EXWAS grouped by the presence of rs201680145.

Figure 3 shows results of PheWAS of NOTCH3 p.Arg1231Cys.

Figure 4 shows a table summarizing the results of PheWAS of NOTCH3 p.Arg1231Cys.

30 Description

Various terms relating to aspects of the present disclosure are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art, unless

- 5 -

otherwise indicated. Other specifically defined terms are to be construed in a manner consistent with the definitions provided herein.

Unless otherwise expressly stated, it is in no way intended that any method or aspect set forth herein be construed as requiring that its steps be performed in a specific order.

5 Accordingly, where a method claim does not specifically state in the claims or descriptions that the steps are to be limited to a specific order, it is in no way intended that an order be inferred, in any respect. This holds for any possible non-expressed basis for interpretation, including matters of logic with respect to arrangement of steps or operational flow, plain meaning derived from grammatical organization or punctuation, or the number or type of aspects
10 described in the specification.

As used herein, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise.

As used herein, the term “about” means that the recited numerical value is approximate and small variations would not significantly affect the practice of the disclosed
15 embodiments. Where a numerical value is used, unless indicated otherwise by the context, the term “about” means the numerical value can vary by $\pm 10\%$ and remain within the scope of the disclosed embodiments.

As used herein, the term “comprising” may be replaced with “consisting” or “consisting essentially of” in particular embodiments as desired.

20 As used herein, the term “isolated”, in regard to a nucleic acid molecule or a polypeptide, means that the nucleic acid molecule or polypeptide is in a condition other than its native environment, such as apart from blood and/or animal tissue. In some embodiments, an isolated nucleic acid molecule or polypeptide is substantially free of other nucleic acid molecules or other polypeptides, particularly other nucleic acid molecules or polypeptides of
25 animal origin. In some embodiments, the nucleic acid molecule or polypeptide can be in a highly purified form, i.e., greater than 95% pure or greater than 99% pure. When used in this context, the term “isolated” does not exclude the presence of the same nucleic acid molecule or polypeptide in alternative physical forms, such as dimers or alternatively phosphorylated or derivatized forms.

30 As used herein, the terms “nucleic acid”, “nucleic acid molecule”, “nucleic acid sequence”, “polynucleotide”, or “oligonucleotide” can comprise a polymeric form of

- 6 -

nucleotides of any length, can comprise DNA and/or RNA, and can be single-stranded, double-stranded, or multiple stranded. One strand of a nucleic acid also refers to its complement.

As used herein, the term “subject” includes any animal, including mammals. Mammals include, but are not limited to, farm animals (such as, for example, horse, cow, pig), companion
5 animals (such as, for example, dog, cat), laboratory animals (such as, for example, mouse, rat, rabbits), and non-human primates (such as, for example, apes and monkeys). In some embodiments, the subject is a human. In some embodiments, the subject is a patient under the care of a physician.

A common variant SNP rs201680145 in the NOTCH3 gene in humans has been
10 identified in accordance with the present disclosure to be associated with an increased risk of developing a cerebrovascular disease, such as CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke. For example, a genetic alteration that changes the cytosine at position 21,944 in the NOTCH3 reference genomic nucleic acid molecule (see, SEQ ID NO:1) to a thymine has been observed to indicate that the subject having such an alteration
15 may have an increased risk of developing a cerebrovascular disease. It is believed that no variants of the NOTCH3 gene or protein have any known significant association, as shown by genome-wide analysis, with a cerebrovascular disease, such as subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke.

In addition, CADASIL was initially reported to be caused by autosomal dominant
20 mutations of the NOTCH3 gene (Joutel et al., Nature, 1996, 383, 707-10), including rs201680145 (Singhal et al., Brain, 2004, 127, 2031-2038). These mutations were believed to lead to an abnormal accumulation of Notch 3 at the cytoplasmic membrane of vascular smooth muscle cells both in cerebral and extracerebral vessels (Joutel et al., J. Clin. Invest., 2000, 105, 597-605) seen as granular osmiophilic deposits on electron microscopy (Ruchoux et al., Acta
25 Neuropathol., 1995, 89, 500-12). In addition, in many cases, leukoencephalopathy follows. All CADASIL pathogenic mutations in NOTCH3 share the common feature of adding or removing a Cys residue from one of 34 EGFR domains in the NOTCH3 extra-cellular domain, where each EGFR normally has 6 Cys residues that are highly conserved in human, mouse and zebrafish NOTCH3. Hundreds of such Cys-add-or-remove variants have been identified. However, the
30 view that the NOTCH3 p.Arg1231Cys mutation is the causative of CADASIL was abandoned as incorrect because of the very high prevalence of this mutation (rs201680145) in South Asians, including, significantly surpassing the frequency that was expected based on CADASIL

- 7 -

prevalence (Rutten et al., Ann. Clin. Transl. Neurol., 2016, 3, 844-853). Altogether, the genetic analyses described herein surprisingly indicate that the NOTCH3 gene and, in particular, a gain-of-function variant in the NOTCH3 gene, associates with an increased risk of developing a cerebrovascular disease, such as CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke. Therefore, subjects that have a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide that have an increased risk of developing a cerebrovascular disease, such as CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke, may be treated such that the cerebrovascular disease is prevented, the symptoms thereof are reduced, and/or development of symptoms is repressed. Accordingly, the present disclosure provides methods of leveraging the identification of such NOTCH3 missense variant nucleic acid molecules in subjects to identify or stratify risk in such subjects of developing a cerebrovascular disease, such as CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke, or to diagnose subjects as having an increased risk of developing a cerebrovascular disease, such as CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke, such that subjects at risk or subjects with active disease may be treated accordingly.

For purposes of the present disclosure, any particular subject can be categorized as having one of three NOTCH3 genotypes: i) NOTCH3 reference; ii) heterozygous for a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide; or iii) homozygous for a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide. A subject is NOTCH3 reference when the subject does not have a copy of a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide. A subject is heterozygous for a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide when the subject has a single copy of a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide. As used herein, a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide is any NOTCH3 nucleic acid molecule (such as, a genomic nucleic acid molecule, an mRNA molecule, or a cDNA molecule) encoding a NOTCH3 polypeptide having a partial gain-of-function, a complete gain-of-function, a predicted partial gain-of-function, or a predicted complete gain-of-function. A subject who has a NOTCH3 polypeptide having a partial gain-of-function (or predicted partial gain-of-function) is hypomorphic for NOTCH3.

- 8 -

In any of the embodiments described herein, the NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide can be any nucleic acid molecule encoding a NOTCH3 Arg1178Cys, Arg1231Cys, or Arg1182Cys. In some embodiments, the NOTCH3 missense variant nucleic acid molecule encodes NOTCH3

- 5 Arg1178Cys, Arg1231Cys, or Arg1182Cys. A subject is homozygous for a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide when the subject has two copies of a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

- 10 In any of the embodiments described herein, the NOTCH3 variant nucleic acid molecules can be any Cys-altering variant (they change from or to a cysteine residue). In some embodiments the Cys-altering variant is located within the 34 EGF-like repeats in the NOTCH3 extracellular domain (defined by Uniprot; see world wide web at “uniprot.org/ blast/?about=Q9UM47[1335-1373]&key=Domain)). In any of the embodiments described herein, the NOTCH3 variant nucleic acid molecules can be any of the variants listed in Table 1
- 15 (ENST00000263388).

Table 1: Cys-Altering Variants

Variant	rsID	HGVS.c	HGVS.p
19:15200890:G:A	rs1189616145	c.16C>T	p.Arg6Cys
19:15200884:G:A		c.22C>T	p.Arg8Cys
19:15200881:G:A		c.25C>T	p.Arg9Cys
19:15200878:G:A		c.28C>T	p.Arg10Cys
19:15200875:G:A		c.31C>T	p.Arg11Cys
19:15200872:G:A		c.34C>T	p.Arg12Cys
19:15200869:G:A		c.37C>T	p.Arg13Cys
19:15197571:A:AG	rs749829137	c.125dupC	p.Cys43fs
19:15197552:A:C		c.145T>G	p.Cys49Gly
19:15197537:G:A		c.160C>T	p.Arg54Cys
19:15197534:A:C		c.163T>G	p.Cys55Gly
19:15197503:C:G		c.194G>C	p.Cys65Ser
19:15192458:A:T		c.259T>A	p.Cys87Ser
19:15192452:C:A		c.265G>T	p.Gly89Cys
19:15192449:G:A		c.268C>T	p.Arg90Cys
19:15192389:G:A	rs775836288	c.328C>T	p.Arg110Cys

- 9 -

19:15192256:C:CAA		c.381_382dupTT	p.Cys128fs
19:15192242:G:A	rs137852642	c.397C>T	p.Arg133Cys
19:15192218:G:A		c.421C>T	p.Arg141Cys
19:15192204:G:GGAGCA		c.430_434dupTGCTC	p.Cys146fs
19:15192182:G:A	rs797045014	c.457C>T	p.Arg153Cys
19:15192155:A:C		c.484T>G	p.Cys162Gly
19:15192135:G:T		c.504C>A	p.Cys168*
19:15192134:G:A	rs28933696	c.505C>T	p.Arg169Cys
19:15192095:G:A	rs28933697	c.544C>T	p.Arg182Cys
19:15192085:C:A		c.554G>T	p.Cys185Phe
19:15192037:C:G		c.602G>C	p.Cys201Ser
19:15192022:C:T		c.617G>A	p.Cys206Tyr
19:15192020:G:A	rs775267348	c.619C>T	p.Arg207Cys
19:15192005:A:T		c.634T>A	p.Cys212Ser
19:15191980:T:C		c.659A>G	p.Tyr220Cys
19:15191974:C:T		c.665G>A	p.Cys222Tyr
19:15191968:C:T		c.671G>A	p.Cys224Tyr
19:15191850:A:T		c.697T>A	p.Cys233Ser
19:15191849:C:T		c.698G>A	p.Cys233Tyr
19:15191848:A:C		c.699T>G	p.Cys233Trp
19:15191829:A:G		c.718T>C	p.Cys240Arg
19:15191828:C:G		c.719G>C	p.Cys240Ser
19:15191827:A:T		c.720T>A	p.Cys240*
19:15191814:A:G		c.733T>C	p.Cys245Arg
19:15191796:A:G		c.751T>C	p.Cys251Arg
19:15191774:T:C		c.773A>G	p.Tyr258Cys
19:15191588:C:T		c.872G>A	p.Cys291Tyr
19:15191565:T:A		c.895A>T	p.Ser299Cys
19:15191529:A:T		c.931T>A	p.Cys311Ser
19:15191507:C:G		c.953G>C	p.Cys318Ser
19:15191507:C:A		c.953G>T	p.Cys318Phe
19:15191466:G:A	rs137852641	c.994C>T	p.Arg332Cys
19:15191456:G:C		c.1004C>G	p.Ser335Cys
19:15191450:T:C		c.1010A>G	p.Tyr337Cys
19:15191446:A:T		c.1014T>A	p.Cys338*
19:15189386:C:T		c.1079G>A	p.Cys360Tyr

- 10 -

19:15189321:C:A		c.1144G>T	p.Gly382Cys
19:15189302:C:T		c.1163G>A	p.Cys388Tyr
19:15189282:A:G		c.1183T>C	p.Cys395Arg
19:15189145:A:G		c.1222T>C	p.Cys408Arg
19:15189112:A:T		c.1255T>A	p.Cys419Ser
19:15189106:G:A		c.1261C>T	p.Arg421Cys
19:15189106:G:A		c.1261C>T	p.Arg421Cys
19:15189083:A:C		c.1284T>G	p.Cys428Trp
19:15189064:A:G		c.1303T>C	p.Cys435Arg
19:15189003:C:T		c.1364G>A	p.Cys455Tyr
19:15188333:T:C		c.1394A>G	p.Tyr465Cys
19:15188301:T:A	rs886054260	c.1426A>T	p.Ser476Cys
19:15188286:C:A	rs1263780227	c.1441G>T	p.Gly481Cys
19:15188276:C:A		c.1451G>T	p.Cys484Phe
19:15188253:T:A		c.1474A>T	p.Ser492Cys
19:15188243:C:T		c.1484G>A	p.Cys495Tyr
19:15187977:A:G		c.1510T>C	p.Cys504Arg
19:15187956:A:G		c.1531T>C	p.Cys511Arg
19:15187955:C:A		c.1532G>T	p.Cys511Phe
19:15187954:G:T		c.1533C>A	p.Cys511*
19:15187940:C:T		c.1547G>A	p.Cys516Tyr
19:15187940:C:A		c.1547G>T	p.Cys516Phe
19:15187922:C:G		c.1565G>C	p.Cys522Ser
19:15187905:C:A		c.1582G>T	p.Gly528Cys
19:15187893:G:A	rs1202763005	c.1594C>T	p.Arg532Cys
19:15187320:C:T		c.1625G>A	p.Cys542Tyr
19:15187315:G:A	rs201118034	c.1630C>T	p.Arg544Cys
19:15187315:G:A	rs201118034	c.1630C>T	p.Arg544Cys
19:15187298:G:C		c.1647C>G	p.Cys549Trp
19:15187296:G:C		c.1649C>G	p.Ser550Cys
19:15187284:C:A		c.1661G>T	p.Cys554Phe
19:15187273:G:A	rs75068032	c.1672C>T	p.Arg558Cys
19:15187273:G:A	rs75068032	c.1672C>T	p.Arg558Cys
19:15187243:A:G		c.1702T>C	p.Cys568Arg
19:15187243:A:C		c.1702T>G	p.Cys568Gly
19:15187242:C:T		c.1703G>A	p.Cys568Tyr

- 11 -

19:15187242:C:CATGAGA A		c.1696_1702dupTTCTCA T	p.Cys568fs
19:15187219:C:A		c.1726G>T	p.Gly576Cys
19:15187213:G:A	rs769773673	c.1732C>T	p.Arg578Cys
19:15187213:G:A	rs769773673	c.1732C>T	p.Arg578Cys
19:15187209:C:T		c.1736G>A	p.Cys579Tyr
19:15187186:G:A	rs754554486	c.1759C>T	p.Arg587Cys
19:15187171:G:A	rs764148985	c.1774C>T	p.Arg592Cys
19:15187129:A:G		c.1816T>C	p.Cys606Arg
19:15187128:C:T		c.1817G>A	p.Cys606Tyr
19:15187128:C:A		c.1817G>T	p.Cys606Phe
19:15187126:G:A	rs777751303	c.1819C>T	p.Arg607Cys
19:15187126:G:A	rs777751303	c.1819C>T	p.Arg607Cys
19:15187121:G:C		c.1824C>G	p.Cys608Trp
19:15186980:A:G		c.1849T>C	p.Cys617Arg
19:15186958:C:T		c.1871G>A	p.Cys624Tyr
19:15186944:A:G		c.1885T>C	p.Cys629Arg
19:15186926:G:A	rs753801611	c.1903C>T	p.Arg635Cys
19:15186911:G:A	rs760768552	c.1918C>T	p.Arg640Cys
19:15186911:G:A	rs760768552	c.1918C>T	p.Arg640Cys
19:15186902:A:C		c.1927T>G	p.Cys643Gly
19:15185671:A:C		c.1960T>G	p.Cys654Gly
19:15185670:C:T		c.1961G>A	p.Cys654Tyr
19:15185670:C:G		c.1961G>C	p.Cys654Ser
19:15185669:A:C		c.1962T>G	p.Cys654Trp
19:15185633:G:T		c.1998C>A	p.Cys666*
19:15185632:C:A	rs376046941	c.1999G>T	p.Gly667Cys
19:15185632:C:A	rs376046941	c.1999G>T	p.Gly667Cys
19:15185619:G:C		c.2012C>G	p.Ser671Cys
19:15185616:C:T		c.2015G>A	p.Cys672Tyr
19:15185616:C:A	rs1480573645	c.2015G>T	p.Cys672Phe
19:15185593:G:A	rs1250956327	c.2038C>T	p.Arg680Cys
19:15185589:C:T		c.2042G>A	p.Cys681Tyr
19:15185521:A:C		c.2110T>G	p.Cys704Gly
19:15185505:C:T		c.2126G>A	p.Cys709Tyr
19:15185502:T:C	rs1328784046	c.2129A>G	p.Tyr710Cys

- 12 -

19:15185404:G:A	rs144163298	c.2149C>T	p.Arg717Cys
19:15185404:G:A	rs144163298	c.2149C>T	p.Arg717Cys
19:15185395:A:G		c.2158T>C	p.Cys720Arg
19:15185394:C:T		c.2159G>A	p.Cys720Tyr
19:15185393:A:T		c.2160T>A	p.Cys720*
19:15185371:G:A	rs1057519101	c.2182C>T	p.Arg728Cys
19:15185358:CTCTGGCTG: C		c.2187_2194delCAGCCA GA	p.Cys729fs
19:15185340:C:T		c.2213G>A	p.Cys738Tyr
19:15185340:C:A		c.2213G>T	p.Cys738Phe
19:15185334:G:C		c.2219C>G	p.Ser740Cys
19:15185325:C:T		c.2228G>A	p.Cys743Tyr
19:15185306:G:C		c.2247C>G	p.Cys749Trp
19:15185280:C:T		c.2273G>A	p.Cys758Tyr
19:15185275:A:G		c.2278T>C	p.Cys760Arg
19:15185274:C:G		c.2279G>C	p.Cys760Ser
19:15185017:G:A	rs532100840	c.2299C>T	p.Arg767Cys
19:15184993:A:G	rs1383763025	c.2323T>C	p.Cys775Arg
19:15184963:G:A	rs1289281166	c.2353C>T	p.Arg785Cys
19:15184929:C:T		c.2387G>A	p.Cys796Tyr
19:15184929:C:G	rs1425871926	c.2387G>C	p.Cys796Ser
19:15184929:C:A	rs1425871926	c.2387G>T	p.Cys796Phe
19:15184928:G:C		c.2388C>G	p.Cys796Trp
19:15184926:G:C		c.2390C>G	p.Ser797Cys
19:15184924:A:C		c.2392T>G	p.Cys798Gly
19:15184923:C:T	rs1325571065	c.2393G>A	p.Cys798Tyr
19:15184922:G:T		c.2394C>A	p.Cys798*
19:15184910:C:A		c.2406G>T	p.Trp802Cys
19:15184442:A:T		c.2419T>A	p.Cys807Ser
19:15184420:C:T		c.2441G>A	p.Cys814Tyr
19:15184419:A:T		c.2442T>A	p.Cys814*
19:15184382:TG:T		c.2478delC	p.Cys826fs
19:15184363:A:C		c.2498T>G	p.Phe833Cys
19:15184356:GC:G		c.2504delG	p.Cys835fs
19:15184339:T:C		c.2522A>G	p.Tyr841Cys
19:15184304:A:G		c.2557T>C	p.Cys853Arg

- 13 -

19:15184303:C:T	rs1253342689	c.2558G>A	p.Cys853Tyr
19:15184302:A:C		c.2559T>G	p.Cys853Trp
19:15181794:G:C		c.2574C>G	p.Cys858Trp
19:15181787:C:A	rs757098265	c.2581G>T	p.Gly861Cys
19:15181778:A:T		c.2590T>A	p.Cys864Ser
19:15181751:A:G		c.2617T>C	p.Cys873Arg
19:15181750:C:A	rs867156576	c.2618G>T	p.Cys873Phe
19:15181744:C:T		c.2624G>A	p.Cys875Tyr
19:15181712:G:A	rs1325474998	c.2656C>T	p.Arg886Cys
19:15181682:A:T		c.2686T>A	p.Cys896Ser
19:15181668:G:GGT		c.2698_2699dupAC	p.Cys901fs
19:15181667:A:T		c.2701T>A	p.Cys901Ser
19:15181648:G:C		c.2720C>G	p.Ser907Cys
19:15181640:A:G		c.2728T>C	p.Cys910Arg
19:15181639:C:T	rs1399374524	c.2729G>A	p.Cys910Tyr
19:15181633:C:G		c.2735G>C	p.Cys912Ser
19:15181621:T:C		c.2747A>G	p.Tyr916Cys
19:15181607:A:G		c.2761T>C	p.Cys921Arg
19:15181606:C:A		c.2762G>T	p.Cys921Phe
19:15181586:AG:A		c.2781delC	p.Cys928fs
19:15181158:A:C	rs749778923	c.2797T>G	p.Cys933Gly
19:15181157:C:T		c.2798G>A	p.Cys933Tyr
19:15181139:C:G	rs1170996533	c.2816G>C	p.Cys939Ser
19:15181138:A:C		c.2817T>G	p.Cys939Trp
19:15181131:C:A	rs777577687	c.2824G>T	p.Gly942Cys
19:15181112:C:A		c.2843G>T	p.Cys948Phe
19:15181104:G:A	rs775964142	c.2851C>T	p.Arg951Cys
19:15181094:T:C		c.2861A>G	p.Tyr954Cys
19:15181080:A:G		c.2875T>C	p.Cys959Arg
19:15181043:C:T		c.2912G>A	p.Cys971Tyr
19:15181004:A:C		c.2951T>G	p.Phe984Cys
19:15181002:G:A		c.2953C>T	p.Arg985Cys
19:15180999:A:G	rs763321998	c.2956T>C	p.Cys986Arg
19:15180999:A:C		c.2956T>G	p.Cys986Gly
19:15180998:C:T		c.2957G>A	p.Cys986Tyr
19:15180975:C:A		c.2980G>T	p.Gly994Cys

- 14 -

19:15180964:G:T		c.2991C>A	p.Cys997*
19:15180812:C:T		c.3011G>A	p.Cys1004Tyr
19:15180807:G:A		c.3016C>T	p.Arg1006Cys
19:15180807:G:A		c.3016C>T	p.Arg1006Cys
19:15180797:C:T	rs1217205469	c.3026G>A	p.Cys1009Tyr
19:15180780:A:G		c.3043T>C	p.Cys1015Arg
19:15180778:G:C		c.3045C>G	p.Cys1015Trp
19:15180761:T:C	rs1167405466	c.3062A>G	p.Tyr1021Cys
19:15180752:C:T		c.3071G>A	p.Cys1024Tyr
19:15180738:T:A		c.3085A>T	p.Ser1029Cys
19:15180732:G:A		c.3091C>T	p.Arg1031Cys
19:15180703:G:T		c.3120C>A	p.Cys1040*
19:15180235:C:T		c.3164G>A	p.Cys1055Tyr
19:15180235:C:G		c.3164G>C	p.Cys1055Ser
19:15180217:C:T	rs1064794216	c.3182G>A	p.Cys1061Tyr
19:15180190:C:G		c.3209G>C	p.Cys1070Ser
19:15180184:C:T	rs1204243987	c.3215G>A	p.Cys1072Tyr
19:15180184:C:A		c.3215G>T	p.Cys1072Phe
19:15180173:G:A	rs1438626607	c.3226C>T	p.Arg1076Cys
19:15180122:A:T		c.3277T>A	p.Cys1093Ser
19:15180103:C:T		c.3296G>A	p.Cys1099Tyr
19:15180101:G:A	rs963416165	c.3298C>T	p.Arg1100Cys
19:15180077:A:G		c.3322T>C	p.Cys1108Arg
19:15180076:C:T		c.3323G>A	p.Cys1108Tyr
19:15180075:A:T		c.3324T>A	p.Cys1108*
19:15179496:A:G		c.3328T>C	p.Cys1110Arg
19:15179468:C:T	rs1266914122	c.3356G>A	p.Cys1119Tyr
19:15179468:C:A		c.3356G>T	p.Cys1119Phe
19:15179432:C:G		c.3392G>C	p.Cys1131Ser
19:15179421:C:A	rs867379493	c.3403G>T	p.Gly1135Cys
19:15179415:A:G		c.3409T>C	p.Cys1137Arg
19:15179414:CA:C		c.3409delT	p.Cys1137fs
19:15179397:G:A	rs60373464	c.3427C>T	p.Arg1143Cys
19:15179397:G:A	rs60373464	c.3427C>T	p.Arg1143Cys
19:15179393:T:C	rs879055341	c.3431A>G	p.Tyr1144Cys
19:15179381:C:G		c.3443G>C	p.Cys1148Ser

- 15 -

19:15179381:C:A		c.3443G>T	p.Cys1148Phe
19:15179274:A:G		c.3469T>C	p.Cys1157Arg
19:15179272:G:C		c.3471C>G	p.Cys1157Trp
19:15179253:A:G		c.3490T>C	p.Cys1164Arg
19:15179250:C:A		c.3493G>T	p.Gly1165Cys
19:15179244:C:A		c.3499G>T	p.Gly1167Cys
19:15179216:C:T		c.3527G>A	p.Cys1176Tyr
19:15179216:C:A		c.3527G>T	p.Cys1176Phe
19:15179197:G:C		c.3546C>G	p.Cys1182Trp
19:15179175:G:A	rs377099118	c.3568C>T	p.Arg1190Cys
19:15179171:C:G	rs1192888680	c.3572G>C	p.Cys1191Ser
19:15179166:A:G		c.3577T>C	p.Cys1193Arg
19:15179165:C:T		c.3578G>A	p.Cys1193Tyr
19:15179142:G:A	rs772172068	c.3601C>T	p.Arg1201Cys
19:15179138:C:T		c.3605G>A	p.Cys1202Tyr
19:15179137:GC:G		c.3605delG	p.Cys1202fs
19:15179137:G:T		c.3606C>A	p.Cys1202*
19:15179115:G:A	rs758961316	c.3628C>T	p.Arg1210Cys
19:15179079:A:G		c.3664T>C	p.Cys1222Arg
19:15179079:A:C	rs199638166	c.3664T>G	p.Cys1222Gly
19:15179079:A:C	rs199638166	c.3664T>G	p.Cys1222Gly
19:15179078:CA:C		c.3664delT	p.Cys1222fs
19:15179078:C:T		c.3665G>A	p.Cys1222Tyr
19:15179052:G:A	rs201680145	c.3691C>T	p.Arg1231Cys
19:15179052:G:A	rs201680145	c.3691C>T	p.Arg1231Cys
19:15179047:G:C	rs1376921184	c.3696C>G	p.Cys1232Trp
19:15178936:G:A	rs769660847	c.3724C>T	p.Arg1242Cys
19:15178912:A:G		c.3748T>C	p.Cys1250Arg
19:15178896:C:T		c.3764G>A	p.Cys1255Tyr
19:15178877:G:C		c.3783C>G	p.Cys1261Trp
19:15178876:G:A		c.3784C>T	p.Arg1262Cys
19:15178864:C:A		c.3796G>T	p.Gly1266Cys
19:15178855:C:A		c.3805G>T	p.Gly1269Cys
19:15178836:C:G		c.3824G>C	p.Cys1275Ser
19:15178830:C:T	rs1339695535	c.3830G>A	p.Cys1277Tyr

- 16 -

19:15178824:TGGGCACA: T		c.3829_3835delTGTGCC	p.Cys1277fs
19:15178082:C:A		c.3846G>T	p.Trp1282Cys
19:15178081:C:A		c.3847G>T	p.Gly1283Cys
19:15178070:G:C		c.3858C>G	p.Cys1286Trp
19:15178057:G:A		c.3871C>T	p.Arg1291Cys
19:15178053:G:C		c.3875C>G	p.Ser1292Cys
19:15178050:C:A		c.3878G>T	p.Cys1293Phe
19:15178017:CA:C		c.3910delT	p.Cys1304fs
19:15178003:G:A		c.3925C>T	p.Arg1309Cys
19:15177990:C:G		c.3938G>C	p.Cys1313Ser
19:15177989:G:C		c.3939C>G	p.Cys1313Trp
19:15177984:C:T	rs1432396805	c.3944G>A	p.Cys1315Tyr
19:15177984:C:A		c.3944G>T	p.Cys1315Phe
19:15177983:G:C	rs1396345163	c.3945C>G	p.Cys1315Trp
19:15177958:A:T	rs1486702985	c.3970T>A	p.Cys1324Ser
19:15177957:C:T		c.3971G>A	p.Cys1324Tyr
19:15177955:G:A		c.3973C>T	p.Arg1325Cys
19:15177898:AG:A		c.4029delC	p.Cys1344fs
19:15177897:CAG:C		c.4029_4030delCT	p.Cys1344fs
19:15177897:CA:C		c.4030delT	p.Cys1344fs
19:15177896:A:C		c.4032T>G	p.Cys1344Trp
19:15177855:A:C		c.4073T>G	p.Phe1358Cys
19:15177850:G:A		c.4078C>T	p.Arg1360Cys
19:15177840:C:T		c.4088G>A	p.Cys1363Tyr
19:15177814:A:G		c.4114T>C	p.Cys1372Arg
19:15177812:G:C		c.4116C>G	p.Cys1372Trp

- For subjects that are genotyped or determined to be heterozygous or homozygous for a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide, such subjects have an increased risk of developing a cerebrovascular
- 5 disease, such as CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke. For subjects that are genotyped or determined to be heterozygous or homozygous for a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide, such subjects can be treated with an agent effective to

- 17 -

treat a cerebrovascular disease such as CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke. For subjects that are genotyped or determined to be heterozygous or homozygous for a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide, such subjects can also or independently be
5 treated with a NOTCH3 agent.

In any of the embodiments described herein, the NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide can be any NOTCH3 nucleic acid molecule (such as, for example, genomic nucleic acid molecule, mRNA molecule, or cDNA molecule) encoding a NOTCH3 polypeptide having a partial gain-of-function, a complete
10 gain-of-function, a predicted partial gain-of-function, or a predicted complete gain-of-function. For example, the NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide can be any nucleic acid molecule encoding NOTCH3 Arg1178Cys, Arg1231Cys, or Arg1182Cys. In some embodiments, the NOTCH3 missense variant nucleic acid molecule encodes NOTCH3 Arg1178Cys. In some embodiments, the NOTCH3 missense variant
15 nucleic acid molecule encodes NOTCH3 Arg1231Cys. In some embodiments, the NOTCH3 missense variant nucleic acid molecule encodes NOTCH3 Arg1182Cys.

In any of the embodiments described herein, the NOTCH3 predicted gain-of-function polypeptide can be any NOTCH3 polypeptide having a partial gain-of-function, a complete gain-of-function, a predicted partial gain-of-function, or a predicted complete gain-of-function. In
20 any of the embodiments described herein, the NOTCH3 predicted gain-of-function polypeptide can be any of the NOTCH3 polypeptides described herein including, for example, NOTCH3 Arg1178Cys, Arg1231Cys, or Arg1182Cys. In some embodiments, the NOTCH3 predicted gain-of-function polypeptide is NOTCH3 Arg1178Cys. In some embodiments, the NOTCH3 predicted gain-of-function polypeptide is NOTCH3 Arg1231Cys. In some embodiments, the NOTCH3
25 predicted gain-of-function polypeptide is NOTCH3 Arg1182Cys.

In any of the embodiments described herein, the cerebrovascular disease is CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke. In any of the embodiments described herein, the cerebrovascular disease is CADASIL. In any of the embodiments described herein, the cerebrovascular disease is subcortical stroke. In any of the
30 embodiments described herein, the cerebrovascular disease is ischemic stroke. In any of the embodiments described herein, the cerebrovascular disease is hemorrhagic stroke. In any of the embodiments described herein, the cerebrovascular disease is parenchymal stroke.

- 18 -

Symptoms of a cerebrovascular disease include, but are not limited to, dizziness, nausea, or vomiting; unusually severe headache; confusion, disorientation or memory loss; numbness, weakness in an arm, leg or the face, especially on one side; abnormal or slurred speech; difficulty with comprehension; loss of vision or difficulty seeing; or loss of balance,
5 coordination or the ability to walk.

The present disclosure provides methods of treating a subject having a cerebrovascular disease, the methods comprising administering a NOTCH3 agent to the subject.

The present disclosure also provides methods of treating a subject having subcortical stroke, the methods comprising administering a NOTCH3 agent to the subject.

10 The present disclosure also provides methods of treating a subject having ischemic stroke, the methods comprising administering a NOTCH3 agent to the subject.

The present disclosure also provides methods of treating a subject having hemorrhagic stroke, the methods comprising administering a NOTCH3 agent to the subject.

The present disclosure also provides methods of treating a subject having parenchymal
15 stroke, the methods comprising administering a NOTCH3 agent to the subject.

The present disclosure also provides methods of treating a subject having CADASIL, the methods comprising administering a NOTCH3 agent to the subject.

In some embodiments, the NOTCH3 agent comprises an inhibitory nucleic acid molecule. In some embodiments, the inhibitory nucleic acid molecule comprises an antisense
20 molecule, a small interfering RNA (siRNA) molecule, or a short hairpin RNA (shRNA) molecule. In some embodiments, the inhibitory nucleic acid molecule comprises an antisense molecule. In some embodiments, the inhibitory nucleic acid molecule comprises an siRNA molecule. In some embodiments, the inhibitory nucleic acid molecule comprises an shRNA molecule. Such inhibitory nucleic acid molecules can be designed to target any region of a NOTCH3 nucleic acid
25 molecule, such as an mRNA molecule. In some embodiments, the inhibitory nucleic acid molecule hybridizes to a sequence within a NOTCH3 genomic nucleic acid molecule or mRNA molecule and decreases expression of the NOTCH3 polypeptide in a cell in the subject. In some embodiments, the NOTCH3 agent comprises an antisense RNA that hybridizes to a NOTCH3 genomic nucleic acid molecule or mRNA molecule and decreases expression of the NOTCH3
30 polypeptide in a cell in the subject. In some embodiments, the NOTCH3 agent comprises an siRNA that hybridizes to a NOTCH3 genomic nucleic acid molecule or mRNA molecule and decreases expression of the NOTCH3 polypeptide in a cell in the subject. In some embodiments,

- 19 -

the NOTCH3 agent comprises an shRNA that hybridizes to a NOTCH3 genomic nucleic acid molecule or mRNA molecule and decreases expression of the NOTCH3 polypeptide in a cell in the subject.

In some embodiments, a representative methodology that can be used for designing an inhibitory nucleic acid molecule for NOTCH3 is described in, for example, U.S. Patent Application Publication No. 2021/0115444, which exemplifies allele-specific suppression. Briefly, the technology is based on oligonucleotides having phosphorothioate backbone linkages, wherein the backbone linkages comprise particular stereoisomers at particular positions. Inclusion of particular chiral structures at a particular location within an oligonucleotide can result in improvement in stability, activity, and specificity of cleavage of target nucleotides. The technology is based on chirally controlled (and/or stereochemically pure) oligonucleotide compositions comprising oligonucleotides defined by having: 1) a common base sequence and length; 2) a common pattern of backbone linkages; 3) a common pattern of backbone chiral centers; and 4) a common pattern of backbone P-modifications. Purity of a chirally controlled oligonucleotide composition can be controlled by stereoselectivity of each coupling step in its preparation process. The initial overall structure of stereochemically pure oligonucleotides (e.g., positioning and chirality of internucleotide linkages) is developed through rational design rules developed in structural studies of substrate-ligand complexes of interest, followed by screening of candidate oligonucleotides. The stereochemically pure preparations are generated in a solid support synthesis process, wherein the solid support is treated with various reagents in several synthesis cycles to achieve the stepwise elongation of a growing oligonucleotide chain with individual nucleotide units. The steps of each cycle include treatment with activating reagent, reaction with a chiral agent, stereospecific condensation, capping of unreacted -OH groups with a protection group, a modification step used to install a modified internucleotidic linkage, and deblocking step to deprotect, for instance, a 5'-OH group for subsequent cycle. The oligonucleotides can be antisense nucleic acid molecules, siRNAs, RNaseH guide sequences, etc. useful for a controlled cleavage of a target nucleic acid polymer, such as a transcript from an allele. The increased specificity for a particular sequence (e.g., allele or SNP) is achieved through overall reduction of cleavage sites targeted by chirally controlled oligonucleotides as compared to unmodified oligonucleotides.

In some embodiments, the NOTCH3 agent comprises a nuclease agent that induces one or more nicks or double-strand breaks at a recognition sequence(s) or a DNA-binding

- 20 -

protein that binds to a recognition sequence within a NOTCH3 genomic nucleic acid molecule. The recognition sequence can be located within a coding region of the NOTCH3 gene, or within regulatory regions that influence the expression of the gene. A recognition sequence of the DNA-binding protein or nuclease agent can be located in an intron, an exon, a promoter, an enhancer, a regulatory region, or any non-protein coding region. The recognition sequence can include or be proximate to the start codon of the NOTCH3 gene. For example, the recognition sequence can be located about 10, about 20, about 30, about 40, about 50, about 100, about 200, about 300, about 400, about 500, or about 1,000 nucleotides from the start codon. As another example, two or more nuclease agents can be used, each targeting a nuclease recognition sequence including or proximate to the start codon. As another example, two nuclease agents can be used, one targeting a nuclease recognition sequence including or proximate to the start codon, and one targeting a nuclease recognition sequence including or proximate to the stop codon, wherein cleavage by the nuclease agents can result in deletion of the coding region between the two nuclease recognition sequences. Any nuclease agent that induces a nick or double-strand break into a desired recognition sequence can be used in the methods and compositions disclosed herein. Any DNA-binding protein that binds to a desired recognition sequence can be used in the methods and compositions disclosed herein.

Suitable nuclease agents and DNA-binding proteins for use herein include, but are not limited to, zinc finger protein or zinc finger nuclease (ZFN) pair, Transcription Activator-Like Effector (TALE) protein or Transcription Activator-Like Effector Nuclease (TALEN), or Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems. The length of the recognition sequence can vary, and includes, for example, recognition sequences that are about 30 to about 36 bp for a zinc finger protein or ZFN pair, about 15 to about 18 bp for each ZFN, about 36 bp for a TALE protein or TALEN, and about 20 bp for a CRISPR/Cas guide RNA.

In some embodiments, CRISPR/Cas systems can be used to modify a NOTCH3 genomic nucleic acid molecule within a cell. The methods and compositions disclosed herein can employ CRISPR-Cas systems by utilizing CRISPR complexes (comprising a guide RNA (gRNA) complexed with a Cas protein) for site-directed cleavage of NOTCH3 nucleic acid molecules.

Cas proteins generally comprise at least one RNA recognition or binding domain that can interact with gRNAs. Cas proteins can also comprise nuclease domains (such as, for example, DNase or RNase domains), DNA binding domains, helicase domains, protein-protein

- 21 -

interaction domains, dimerization domains, and other domains. Suitable Cas proteins include, for example, a wild type Cas9 protein and a wild type Cpf1 protein (such as, for example, FnCpf1). A Cas protein can have full cleavage activity to create a double-strand break in a NOTCH3 genomic nucleic acid molecule or it can be a nickase that creates a single-strand break

5 in a NOTCH3 genomic nucleic acid molecule. Additional examples of Cas proteins include, but are not limited to, Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas5e (CasD), Cas6, Cas6e, Cas6f, Cas7, Cas8a1, Cas8a2, Cas8b, Cas8c, Cas9 (Csn1 or Csx12), Cas10, Cas10d, CasF, CasG, CasH, Csy1, Csy2, Csy3, Cse1 (CasA), Cse2 (CasB), Cse3 (CasE), Cse4 (CasC), Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14,

10 Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, and Cu1966, and homologs or modified versions thereof. Cas proteins can also be operably linked to heterologous polypeptides as fusion proteins. For example, a Cas protein can be fused to a cleavage domain, an epigenetic modification domain, a transcriptional activation domain, or a transcriptional repressor domain. Cas proteins can be provided in any form. For example, a Cas protein can be

15 provided in the form of a protein, such as a Cas protein complexed with a gRNA. Alternately, a Cas protein can be provided in the form of a nucleic acid molecule encoding the Cas protein, such as an RNA or DNA.

In some embodiments, targeted genetic modifications of a NOTCH3 genomic nucleic acid molecules can be generated by contacting a cell with a Cas protein and one or more gRNAs

20 that hybridize to one or more gRNA recognition sequences within a target genomic locus in the NOTCH3 genomic nucleic acid molecule. For example, a gRNA recognition sequence can be located within a region of SEQ ID NO:1. The gRNA recognition sequence can also include or be proximate to a position corresponding to position 21,944 according to SEQ ID NO:1. For example, the gRNA recognition sequence can be located about 1000, about 500, about 400,

25 about 300, about 200, about 100, about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 15, about 10, or about 5 nucleotides from a position corresponding to position 21,944 according to SEQ ID NO:1. The gRNA recognition sequence can include or be proximate to the start codon or the stop codon of a NOTCH3 genomic nucleic acid molecule. For example, the gRNA recognition sequence can be located about 10, about 20, about 30, about 40, about

30 50, about 100, about 200, about 300, about 400, about 500, or about 1,000 nucleotides from the start codon or the stop codon.

- 22 -

The gRNA recognition sequences within a target genomic locus in a NOTCH3 genomic nucleic acid molecule are located near a Protospacer Adjacent Motif (PAM) sequence, which is a 2-6 base pair DNA sequence immediately following the DNA sequence targeted by the Cas9 nuclease. The canonical PAM is the sequence 5'-NGG-3' where "N" is any nucleobase followed by two guanine ("G") nucleobases. gRNAs can transport Cas9 to anywhere in the genome for gene editing, but no editing can occur at any site other than one at which Cas9 recognizes a PAM. In addition, 5'-NGA-3' can be a highly efficient non-canonical PAM for human cells. Generally, the PAM is about 2 to about 6 nucleotides downstream of the DNA sequence targeted by the gRNA. The PAM can flank the gRNA recognition sequence. In some embodiments, the gRNA recognition sequence can be flanked on the 3' end by the PAM. In some embodiments, the gRNA recognition sequence can be flanked on the 5' end by the PAM. For example, the cleavage site of Cas proteins can be about 1 to about 10 base pairs, about 2 to about 5 base pairs, or 3 base pairs upstream or downstream of the PAM sequence. In some embodiments (such as when Cas9 from *S. pyogenes* or a closely related Cas9 is used), the PAM sequence of the non-complementary strand can be 5'-NGG-3', where N is any DNA nucleotide and is immediately 3' of the gRNA recognition sequence of the non-complementary strand of the target DNA. As such, the PAM sequence of the complementary strand would be 5'-CCN-3', where N is any DNA nucleotide and is immediately 5' of the gRNA recognition sequence of the complementary strand of the target DNA.

A gRNA is an RNA molecule that binds to a Cas protein and targets the Cas protein to a specific location within a NOTCH3 genomic nucleic acid molecule. An exemplary gRNA is a gRNA effective to direct a Cas enzyme to bind to or cleave a NOTCH3 genomic nucleic acid molecule, wherein the gRNA comprises a DNA-targeting segment that hybridizes to a gRNA recognition sequence within the NOTCH3 genomic nucleic acid molecule that includes or is proximate to a position corresponding to position 21,944 according to SEQ ID NO:1. For example, a gRNA can be selected such that it hybridizes to a gRNA recognition sequence that is located about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 200, about 300, about 400, about 500, or about 1,000 nucleotides from a position corresponding to position 21,944 according to SEQ ID NO:1. Other exemplary gRNAs comprise a DNA-targeting segment that hybridizes to a gRNA recognition sequence present within a NOTCH3 genomic nucleic acid molecule that includes or is proximate to the start codon or the stop codon. For example, a gRNA can be selected such that it hybridizes to a gRNA

- 23 -

recognition sequence that is located about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 200, about 300, about 400, about 500, or about 1,000 nucleotides from the start codon or located about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 200, about 300, about 400, about 500, or about 1,000 nucleotides from the stop codon. Suitable gRNAs can comprise from about 17 to about 25 nucleotides, from about 17 to about 23 nucleotides, from about 18 to about 22 nucleotides, or from about 19 to about 21 nucleotides. In some embodiments, the gRNAs comprise 20 nucleotides.

Examples of suitable gRNA recognition sequences located within the NOTCH3 reference gene are set forth in Table 2 as SEQ ID NOs:29-43.

Table 2: Guide RNA Recognition Sequences Near NOTCH3 Variation(s)

Strand	gRNA Recognition Sequence	SEQ ID NO:
+	ATACACTGGTTTGCCTGCGAGG	29
+	TCTCAGGTAAGCGTTGGCGAAGG	30
+	CTACATGCTCCCGCTCGCTCAGG	31
+	CTCAGGTAAGCGTTGGCGAAGGG	32
+	TCAGGTAAGCGTTGGCGAAGGGG	33
+	GACATCAATGAGTGTGCTCAGG	34
-	AGTCCCGGGTGTGTGCCGCGTGG	35
+	CTGGCTTCTCAGGTAAGCGTTGG	36
-	GCATGTAGATCAGCCACAATGGG	37
-	GACAGGACAGTCTGACAGCGAGG	38
-	ATGTAGATCAGCCACAATGGGGG	39
-	AGCATGTAGATCAGCCACAATGG	40
-	ACAGCGAGGACCTGAGCGAGCGG	41
+	GTAAGCGTTGGCGAAGGGGCTGG	42
-	CATGTAGATCAGCCACAATGGGG	43

The Cas protein and the gRNA form a complex, and the Cas protein cleaves the target NOTCH3 genomic nucleic acid molecule. The Cas protein can cleave the nucleic acid molecule at a site within or outside of the nucleic acid sequence present in the target NOTCH3 genomic

- 24 -

nucleic acid molecule to which the DNA-targeting segment of a gRNA will bind. For example, formation of a CRISPR complex (comprising a gRNA hybridized to a gRNA recognition sequence and complexed with a Cas protein) can result in cleavage of one or both strands in or near (such as, for example, within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the nucleic acid sequence present in the NOTCH3 genomic nucleic acid molecule to which a DNA-targeting segment of a gRNA will bind.

Such methods can result, for example, in a NOTCH3 genomic nucleic acid molecule in which a region of SEQ ID NO:2 is disrupted, the start codon is disrupted, the stop codon is disrupted, or the coding sequence is disrupted or deleted. Optionally, the cell can be further contacted with one or more additional gRNAs that hybridize to additional gRNA recognition sequences within the target genomic locus in the NOTCH3 genomic nucleic acid molecule. By contacting the cell with one or more additional gRNAs (such as, for example, a second gRNA that hybridizes to a second gRNA recognition sequence), cleavage by the Cas protein can create two or more double-strand breaks or two or more single-strand breaks.

In some embodiments, the NOTCH3 agent is an anti-aggregate antibody or agonist antibody that increase processing. Such antibodies include, but are not limited to, 5E1 antibody (or a humanized version thereof) (Ghezali et al., *Ann. Neurol.* 2018, 84, 246-259) and A13 antibody (Machuca-Parra et al., *J. Exp. Med.*, 2017, 214, 2271-2282).

In some embodiments, the methods of treatment further comprise detecting the presence or absence of a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide in a biological sample from the subject. As used throughout the present disclosure, "a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide" is any NOTCH3 nucleic acid molecule (such as, for example, genomic nucleic acid molecule, mRNA molecule, or cDNA molecule) encoding a NOTCH3 polypeptide having a partial gain-of-function, a complete gain-of-function, a predicted partial gain-of-function, or a predicted complete gain-of-function.

The present disclosure also provides methods of treating a subject with a therapeutic agent that treats or inhibits a cerebrovascular disease. In some embodiments, the subject has a cerebrovascular disease. In some embodiments, the methods comprise determining whether the subject has a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide by obtaining or having obtained a biological sample from the subject, and performing or having performed a sequencing analysis on the biological sample to

- 25 -

determine if the subject has a genotype comprising the NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide. The methods further comprise administering or continuing to administer the therapeutic agent that treats or inhibits a cerebrovascular disease in a standard dosage amount to a subject that is NOTCH3 reference, and administering a NOTCH3 agent to the subject. Alternately, the methods further comprise administering or continuing to administer the therapeutic agent that treats or inhibits a cerebrovascular disease in an amount that is the same as or greater than a standard dosage amount to a subject that is heterozygous or homozygous for the NOTCH3 missense variant nucleic acid molecule, and administering a NOTCH3 agent to the subject. The presence of a genotype having the NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide indicates the subject has an increased risk of developing a cerebrovascular disease.

Detecting the presence or absence of a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide in a biological sample from a subject and/or determining whether a subject has a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide can be carried out by any of the methods described herein. In some embodiments, these methods can be carried out *in vitro*. In some embodiments, these methods can be carried out *in situ*. In some embodiments, these methods can be carried out *in vivo*. In any of these embodiments, the nucleic acid molecule can be present within a cell obtained from the subject.

The present disclosure also provides methods of treating a subject with a therapeutic agent that treats or inhibits a cerebrovascular disease. In some embodiments, the subject has a cerebrovascular disease. In some embodiments, the method comprises determining whether the subject has a NOTCH3 predicted gain-of-function polypeptide by obtaining or having obtained a biological sample from the subject, and performing or having performed an assay on the biological sample to determine if the subject has a NOTCH3 predicted gain-of-function polypeptide. When the subject does not have a NOTCH3 predicted gain-of-function polypeptide, the therapeutic agent that treats or inhibits a cerebrovascular disease is administered or continued to be administered to the subject in a standard dosage amount. When the subject has a NOTCH3 predicted gain-of-function polypeptide, the therapeutic agent that treats or inhibits a cerebrovascular disease is administered or continued to be administered to the subject in an amount that is the same as or greater than a standard dosage

- 26 -

amount. The presence of a NOTCH3 predicted gain-of-function polypeptide indicates the subject has an induced risk of developing a cerebrovascular disease. In some embodiments, the subject has a NOTCH3 predicted gain-of-function polypeptide. In some embodiments, the subject does not have a NOTCH3 predicted gain-of-function polypeptide.

5 Detecting the presence or absence of a NOTCH3 predicted gain-of-function polypeptide in a biological sample from a subject and/or determining whether a subject has a NOTCH3 predicted gain-of-function polypeptide can be carried out by any of the methods described herein. In some embodiments, these methods can be carried out *in vitro*. In some
10 embodiments, these methods can be carried out *in situ*. In some embodiments, these methods can be carried out *in vivo*. In any of these embodiments, the polypeptide can be present within a cell obtained from the subject.

 Examples of therapeutic agents that treat or inhibit a cerebrovascular disease include, but are not limited to: tissue plasminogen activator (tPA); anticoagulants such as apixaban, dabigatran, edoxaban, rivaroxaban, and warfarin; blood pressure medications such as diuretics
15 (e.g., bumetanide, ethacrynic acid, furosemide, and torsemide), ACE inhibitors (e.g., benazepril, captopril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, and trandolapril), beta blockers (e.g., acebutolol, atenolol, bisoprolol, metoprolol, nadolol, nebivolol, and propranolol); calcium channel blockers (e.g., amlodipine, felodipine, isradipine, nifedipine, nimodipine, and nitrendipine); and cholesterol-lowering medications
20 such as statins (e.g., atorvastatin, Fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin), or any combination thereof.

 In some embodiments, the dose of the therapeutic agents that treat or inhibit a cerebrovascular disease can be increased by about 10%, by about 20%, by about 30%, by about 40%, by about 50%, by about 60%, by about 70%, by about 80%, or by about 90% for subjects
25 that are heterozygous or homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide (i.e., a greater amount than the standard dosage amount) compared to subjects that are NOTCH3 reference (who may receive a standard dosage amount). In some embodiments, the dose of the therapeutic agents that treat or inhibit a cerebrovascular disease can be increased by about 10%, by about 20%, by about
30 30%, by about 40%, or by about 50%. In addition, the dose of therapeutic agents that treat or inhibit a cerebrovascular disease in subjects that are heterozygous or homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-

- 27 -

function polypeptide can be administered more frequently compared to subjects that are NOTCH3 reference.

In some embodiments, the dose of the therapeutic agents that treat or inhibit a cerebrovascular disease can be increased by about 10%, by about 20%, by about 30%, by about 5 40%, by about 50%, by about 60%, by about 70%, by about 80%, or by about 90% for subjects that are homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide compared to subjects that are heterozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide. In some embodiments, the dose of the therapeutic agents that treat or 10 inhibit a cerebrovascular disease can be increased by about 10%, by about 20%, by about 30%, by about 40%, or by about 50%. In addition, the dose of therapeutic agents that treat or inhibit a cerebrovascular disease in subjects that are homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide can be administered more frequently compared to subjects that heterozygous for a NOTCH3 missense 15 variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide.

Administration of the therapeutic agents that treat or inhibit a cerebrovascular disease and/or NOTCH3 agents can be repeated, for example, after one day, two days, three days, five days, one week, two weeks, three weeks, one month, five weeks, six weeks, seven weeks, eight weeks, two months, or three months. The repeated administration can be at the same dose or 20 at a different dose. The administration can be repeated once, twice, three times, four times, five times, six times, seven times, eight times, nine times, ten times, or more. For example, according to certain dosage regimens a subject can receive therapy for a prolonged period of time such as, for example, 6 months, 1 year, or more.

Administration of the therapeutic agents that treat or inhibit a cerebrovascular disease 25 and/or NOTCH3 agents can occur by any suitable route including, but not limited to, parenteral, intravenous, oral, subcutaneous, intra-arterial, intracranial, intrathecal, intraperitoneal, topical, intranasal, or intramuscular. Pharmaceutical compositions for administration are desirably sterile and substantially isotonic and manufactured under GMP conditions. Pharmaceutical compositions can be provided in unit dosage form (i.e., the dosage for a single administration). 30 Pharmaceutical compositions can be formulated using one or more physiologically and pharmaceutically acceptable carriers, diluents, excipients or auxiliaries. The formulation depends on the route of administration chosen. The term "pharmaceutically acceptable" means

- 28 -

that the carrier, diluent, excipient, or auxiliary is compatible with the other ingredients of the formulation and not substantially deleterious to the recipient thereof.

The terms “treat”, “treating”, and “treatment” and “prevent”, “preventing”, and “prevention” as used herein, refer to eliciting the desired biological response, such as a therapeutic and prophylactic effect, respectively. In some embodiments, a therapeutic effect comprises one or more of a decrease/reduction in a cerebrovascular disease, a decrease/reduction in the severity of a cerebrovascular disease (such as, for example, a reduction or inhibition of development of a cerebrovascular disease), a decrease/reduction in symptoms and cerebrovascular disease-related effects, delaying the onset of symptoms and cerebrovascular disease-related effects, reducing the severity of symptoms of cerebrovascular disease-related effects, reducing the severity of an acute episode, reducing the number of symptoms and cerebrovascular disease-related effects, reducing the latency of symptoms and cerebrovascular disease-related effects, an amelioration of symptoms and cerebrovascular disease-related effects, reducing secondary symptoms, reducing secondary infections, preventing relapse to a cerebrovascular disease, decreasing the number or frequency of relapse episodes, increasing latency between symptomatic episodes, increasing time to sustained progression, expediting remission, inducing remission, augmenting remission, speeding recovery, or increasing efficacy of or decreasing resistance to alternative therapeutics, and/or an increased survival time of the affected host animal, following administration of the agent or composition comprising the agent. A prophylactic effect may comprise a complete or partial avoidance/inhibition or a delay of a cerebrovascular disease development/progression (such as, for example, a complete or partial avoidance/inhibition or a delay), and an increased survival time of the affected host animal, following administration of a therapeutic protocol. Treatment of a cerebrovascular disease encompasses the treatment of subjects already diagnosed as having any form of a cerebrovascular disease at any clinical stage or manifestation, the delay of the onset or evolution or aggravation or deterioration of the symptoms or signs of a cerebrovascular disease, and/or preventing and/or reducing the severity of a cerebrovascular disease.

The present disclosure also provides methods of identifying a subject having an increased risk for developing a cerebrovascular disease. In some embodiments, the method comprises determining or having determined in a biological sample obtained from the subject the presence or absence of a NOTCH3 missense variant nucleic acid molecule encoding the

- 29 -

NOTCH3 predicted gain-of-function polypeptide (such as a genomic nucleic acid molecule, mRNA molecule, and/or cDNA molecule). When the subject lacks a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide (i.e., the subject is genotypically categorized as a NOTCH3 reference), then the subject does not have an increased risk for developing a cerebrovascular disease. When the subject has a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide (i.e., the subject is heterozygous or homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide), then the subject has an increased risk for developing a cerebrovascular disease.

10 Having two copies of a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide may render the subject to have a greater risk of developing a cerebrovascular disease than having a single copy of a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide. Without intending to be limited to any particular theory or mechanism of action, it is believed
15 that a single copy of a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide (i.e., heterozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide) renders the subject to have a greater risk of developing a cerebrovascular disease compared to a subject who is NOTCH3 reference, and it is also believed that having two copies of a NOTCH3
20 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide (i.e., homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide) may render the subject to have an even greater risk of developing a cerebrovascular disease, relative to a subject with a single copy.

 Determining whether a subject has a NOTCH3 missense variant nucleic acid molecule
25 encoding the NOTCH3 predicted gain-of-function polypeptide in a biological sample from the subject and/or determining whether a subject has a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide can be carried out by any of the methods described herein. In some embodiments, these methods can be carried out *in vitro*. In some embodiments, these methods can be carried out *in situ*. In some
30 embodiments, these methods can be carried out *in vivo*. In any of these embodiments, the nucleic acid molecule can be present within a cell obtained from the subject.

- 30 -

In some embodiments, when a subject is identified as having an increased risk of developing a cerebrovascular disease, the subject is further treated with a therapeutic agent that treats or inhibits a cerebrovascular disease, and/or a NOTCH3 agent, as described herein. For example, when the subject is heterozygous or homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide, and
5 therefore has an increased risk for developing a cerebrovascular disease, the subject is administered a therapeutic agent that treats or inhibits a cerebrovascular disease in a dosage amount that is the same as or greater than a standard dosage amount, and/or a NOTCH3 agent. In some embodiments, when the subject is homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide, the subject is
10 administered a therapeutic agent that treats or inhibits a cerebrovascular disease in a dosage amount that is the same as or greater than the dosage amount that is administered to a subject that is heterozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide. In some embodiments, the subject is NOTCH3
15 reference. In some embodiments, the subject is heterozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide. In some embodiments, the subject is homozygous for a NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide.

The present disclosure also provides methods of detecting the presence or absence of
20 a NOTCH3 missense variant genomic nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide in a biological sample from a subject, and/or a NOTCH3 missense variant mRNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide in a biological sample from a subject, and/or a NOTCH3 missense variant cDNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide produced from an mRNA molecule in a
25 biological sample from a subject. It is understood that gene sequences within a population and mRNA molecules encoded by such genes can vary due to polymorphisms such as single-nucleotide polymorphisms. The sequences provided herein for the NOTCH3 variant genomic nucleic acid molecule, NOTCH3 variant mRNA molecule, and NOTCH3 variant cDNA molecule are only exemplary sequences. Other sequences for the NOTCH3 variant genomic nucleic acid
30 molecule, variant mRNA molecule, and variant cDNA molecule are also possible.

The biological sample can be derived from any cell, tissue, or biological fluid from the subject. The biological sample may comprise any clinically relevant tissue such as, for example,

- 31 -

a bone marrow sample, a tumor biopsy, a fine needle aspirate, or a sample of bodily fluid, such as blood, gingival crevicular fluid, plasma, serum, lymph, ascitic fluid, cystic fluid, or urine. In some embodiments, the sample comprises a buccal swab. The biological sample used in the methods disclosed herein can vary based on the assay format, nature of the detection method, and the tissues, cells, or extracts that are used as the sample. A biological sample can be processed differently depending on the assay being employed. For example, when detecting any NOTCH3 variant nucleic acid molecule, preliminary processing designed to isolate or enrich the biological sample for the NOTCH3 variant nucleic acid molecule can be employed. A variety of techniques may be used for this purpose. When detecting the level of any NOTCH3 variant mRNA molecule, different techniques can be used to enrich the biological sample with mRNA molecules. Various methods to detect the presence or level of an mRNA molecule or the presence of a particular variant genomic DNA locus can be used.

In some embodiments, detecting a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide in a subject comprises assaying or analyzing a biological sample obtained from the subject to determine whether a NOTCH3 missense variant genomic nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide in the biological sample, a NOTCH3 missense variant mRNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide in the biological sample, and/or a NOTCH3 missense variant cDNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide produced from an mRNA molecule in the biological sample, comprises one or more variations that cause a gain-of-function (partial or complete) or are predicted to cause a gain-of-function (partial or complete).

In some embodiments, the methods of detecting the presence or absence of a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide (such as, for example, a genomic nucleic acid molecule, an mRNA molecule, and/or a cDNA molecule produced from an mRNA molecule) in a subject, comprise performing an assay on a biological sample obtained from the subject. The assay determines whether a nucleic acid molecule in the biological sample comprises a particular nucleotide sequence.

In some embodiments, the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof.

- 32 -

In some embodiments, the nucleotide sequence of the NOTCH3 mRNA molecule comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 mRNA molecule comprises a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 mRNA molecule comprises a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 mRNA molecule comprises a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 mRNA molecule comprises a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 mRNA molecule comprises a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or the complement thereof.

In some embodiments, the nucleotide sequence of the NOTCH3 cDNA molecule comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 cDNA molecule comprises a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 cDNA molecule comprises a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 cDNA molecule comprises a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 cDNA molecule comprises a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21, or the complement thereof. In some embodiments, the nucleotide sequence of the NOTCH3 cDNA

- 33 -

molecule comprises a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22, or the complement thereof.

In some embodiments, the biological sample comprises a cell or cell lysate. Such methods can further comprise, for example, obtaining a biological sample from the subject comprising a NOTCH3 genomic nucleic acid molecule or mRNA molecule, and if mRNA, optionally reverse transcribing the mRNA into cDNA. Such assays can comprise, for example determining the identity of these positions of the particular NOTCH3 nucleic acid molecule. In some embodiments, the method is an *in vitro* method.

In some embodiments, the determining step, detecting step, or sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule, the NOTCH3 mRNA molecule, or the NOTCH3 cDNA molecule produced from the mRNA molecule in the biological sample, wherein the sequenced portion comprises one or more variations that cause a gain-of-function (partial or complete) or are predicted to cause a gain-of-function (partial or complete).

In some embodiments, the determining step, detecting step, or sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

In some embodiments, the determining step, detecting step, or sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA molecule in the biological sample, or cDNA molecule produced therefrom, wherein the sequenced portion comprises a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; and/or position 3,781 according to SEQ ID NO:18, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises: a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

- 34 -

In some embodiments, the determining step, detecting step, or sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA molecule in the biological sample, or cDNA molecule produced therefrom, wherein the sequenced portion comprises a position corresponding to: position 3,767 according to SEQ ID NO:9, or the complement thereof; and/or position 3,767 according to SEQ ID NO:19, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises: a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

In some embodiments, the determining step, detecting step, or sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA molecule in the biological sample, or cDNA molecule produced therefrom, wherein the sequenced portion comprises a position corresponding to: position 3,532 according to SEQ ID NO:10, or the complement thereof; and/or position 3,532 according to SEQ ID NO:20, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises: a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

In some embodiments, the determining step, detecting step, or sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA molecule in the biological sample, or cDNA molecule produced therefrom, wherein the sequenced portion comprises a position corresponding to: position 3,769 according to SEQ ID NO:11, or the complement thereof; and/or position 3,769 according to SEQ ID NO:21, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises: a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

In some embodiments, the determining step, detecting step, or sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA

- 35 -

molecule in the biological sample, or cDNA molecule produced therefrom, wherein the sequenced portion comprises a position corresponding to: position 3,544 according to SEQ ID NO:12, or the complement thereof; and/or position 3,544 according to SEQ ID NO:22, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises: a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 cDNA molecule produced from the mRNA molecule in the biological sample, wherein the sequenced

- 36 -

portion comprises a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; or position 3,532 according to SEQ ID NO:20, or the complement thereof. When the sequenced portion of the NOTCH3 nucleic acid molecule in the biological sample comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22, then the NOTCH3 nucleic acid molecule in the biological sample is a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

10 In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule that is proximate to a position corresponding to position 21,944 according to SEQ ID NO:2; b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule corresponding to position 21,944 according to SEQ ID NO:2; and c) determining whether the extension product of the primer comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2.

20 In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 mRNA molecule that is proximate to a position corresponding to position 3,781 according to SEQ ID NO:8; and/or cDNA molecule that is proximate to a position corresponding to position 3,781 according to SEQ ID NO:18; b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 mRNA molecule corresponding to position 3,781 according to SEQ ID NO:8; and/or cDNA molecule corresponding to position 3,781 according to SEQ ID NO:18; and c) determining whether the extension product of the primer comprises: a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, and/or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18.

30 In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 mRNA molecule that is proximate to a position corresponding to position 3,767 according to SEQ ID NO:9; and/or cDNA molecule that is

- 37 -

proximate to a position corresponding to position 3,767 according to SEQ ID NO:19; b)
extending the primer at least through the position of the nucleotide sequence of the NOTCH3
mRNA molecule corresponding to position 3,767 according to SEQ ID NO:9; and/or cDNA
molecule corresponding to position 3,767 according to SEQ ID NO:19; and c) determining
5 whether the extension product of the primer comprises: a uracil at a position corresponding to
position 3,767 according to SEQ ID NO:9, and/or a thymine at a position corresponding to
position 3,767 according to SEQ ID NO:19.

In some embodiments, the determining step, detecting step, or sequencing analysis
comprises: a) contacting the biological sample with a primer hybridizing to a portion of the
10 nucleotide sequence of the NOTCH3 mRNA molecule that is proximate to a position
corresponding to position 3,532 according to SEQ ID NO:10; and/or cDNA molecule that is
proximate to a position corresponding to position 3,532 according to SEQ ID NO:20; b)
extending the primer at least through the position of the nucleotide sequence of the NOTCH3
mRNA molecule corresponding to position 3,532 according to SEQ ID NO:10; and/or cDNA
15 molecule corresponding to position 3,532 according to SEQ ID NO:20; and c) determining
whether the extension product of the primer comprises: a uracil at a position corresponding to
position 3,532 according to SEQ ID NO:10, and/or a thymine at a position corresponding to
position 3,532 according to SEQ ID NO:20.

In some embodiments, the determining step, detecting step, or sequencing analysis
20 comprises: a) contacting the biological sample with a primer hybridizing to a portion of the
nucleotide sequence of the NOTCH3 mRNA molecule that is proximate to a position
corresponding to position 3,769 according to SEQ ID NO:11; and/or cDNA molecule that is
proximate to a position corresponding to position 3,769 according to SEQ ID NO:21; b)
extending the primer at least through the position of the nucleotide sequence of the NOTCH3
25 mRNA molecule corresponding to position 3,769 according to SEQ ID NO:11; and/or cDNA
molecule corresponding to position 3,769 according to SEQ ID NO:21; and c) determining
whether the extension product of the primer comprises: a uracil at a position corresponding to
position 3,769 according to SEQ ID NO:11, and/or a thymine at a position corresponding to
position 3,769 according to SEQ ID NO:21.

30 In some embodiments, the determining step, detecting step, or sequencing analysis
comprises: a) contacting the biological sample with a primer hybridizing to a portion of the
nucleotide sequence of the NOTCH3 mRNA molecule that is proximate to a position

- 38 -

corresponding to position 3,544 according to SEQ ID NO:12; and/or cDNA molecule that is proximate to a position corresponding to position 3,544 according to SEQ ID NO:22; b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 mRNA molecule corresponding to position 3,544 according to SEQ ID NO:12; and/or cDNA molecule corresponding to position 3,544 according to SEQ ID NO:22; and c) determining whether the extension product of the primer comprises: a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, and/or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 mRNA molecule that is proximate to a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10; position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12; b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 mRNA molecule corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12; and c) determining whether the extension product of the primer comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 cDNA molecule that is proximate to a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22; b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 cDNA molecule corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, position or 3,544 according to SEQ ID NO:22; and c) determining whether the extension product of the primer comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, position

- 39 -

3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22.

In some embodiments, the assay comprises sequencing the entire nucleic acid molecule. In some embodiments, only a NOTCH3 genomic nucleic acid molecule is analyzed. In
5 some embodiments, only a NOTCH3 mRNA is analyzed. In some embodiments, only a NOTCH3 cDNA obtained from NOTCH3 mRNA is analyzed.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the amplified portion comprises a thymine at a position
10 corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; b) labeling the amplified nucleic acid molecule with a detectable label; c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a
15 thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; and d) detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the amplified portion comprises: a uracil at a position
20 corresponding to position 3,781 according to SEQ ID NO:8, or the complement thereof; and/or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18, or the complement thereof; b) labeling the amplified nucleic acid molecule with a detectable label; c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes
25 under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising: a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or the complement thereof; and/or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18, or the complement thereof; and d) detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis
30 comprises: a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the amplified portion comprises: a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or the complement thereof; and/or

- 40 -

a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19, or the complement thereof; b) labeling the amplified nucleic acid molecule with a detectable label; c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising: a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or the complement thereof; and/or a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19, or the complement thereof; and d) detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the amplified portion comprises: a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or the complement thereof; and/or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20, or the complement thereof; b) labeling the amplified nucleic acid molecule with a detectable label; c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising: a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or the complement thereof; and/or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20, or the complement thereof; and d) detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the amplified portion comprises: a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or the complement thereof; and/or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21, or the complement thereof; b) labeling the amplified nucleic acid molecule with a detectable label; c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising: a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or the complement thereof; and/or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21, or the complement thereof; and d) detecting the detectable label.

- 41 -

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the amplified portion comprises: a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or the complement thereof; and/or
5 a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22, or the complement thereof; b) labeling the amplified nucleic acid molecule with a detectable label; c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule
10 comprising: a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or the complement thereof; and/or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22, or the complement thereof; and d) detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: a) amplifying at least a portion of the nucleic acid molecule that encodes the
15 NOTCH3 polypeptide, wherein the amplified portion comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof;
20 b) labeling the amplified nucleic acid molecule with a detectable label; c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the
25 complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; and d) detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis
30 comprises: a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the amplified portion comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof;

- 42 -

position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof; b) labeling the amplified nucleic acid molecule with a detectable label; c) contacting the labeled
5 nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof;
10 position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof; and d) detecting the detectable label.

In some embodiments, the nucleic acid molecule is mRNA and the determining step further comprises reverse-transcribing the mRNA into a cDNA prior to the amplifying step.

15 In some embodiments, the determining step, detecting step, or sequencing analysis comprises: contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to
20 position 21,944 according to SEQ ID NO:2, or the complement thereof; and detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a
25 nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or the complement thereof; and/or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18, or the complement thereof; and detecting the detectable label.

30 In some embodiments, the determining step, detecting step, or sequencing analysis comprises: contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a

- 43 -

nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising: a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or the complement thereof; and/or a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19, or the complement thereof; and
5 detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of
10 the amplified nucleic acid molecule comprising: a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or the complement thereof; and/or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20, or the complement thereof; and detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis
15 comprises: contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising: a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or the complement thereof; and/or a thymine at a position
20 corresponding to position 3,769 according to SEQ ID NO:21, or the complement thereof; and detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a
25 nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising: a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or the complement thereof; and/or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22, or the complement thereof; and detecting the detectable label.

30 In some embodiments, the determining step, detecting step, or sequencing analysis comprises: contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a

- 44 -

nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; and detecting the detectable label.

In some embodiments, the determining step, detecting step, or sequencing analysis comprises: contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof; and detecting the detectable label.

Alteration-specific polymerase chain reaction techniques can be used to detect mutations such as SNPs in a nucleic acid sequence. Alteration-specific primers can be used because the DNA polymerase will not extend when a mismatch with the template is present.

In some embodiments, the nucleic acid molecule in the sample is mRNA and the mRNA is reverse-transcribed into a cDNA prior to the amplifying step. In some embodiments, the nucleic acid molecule is present within a cell obtained from the subject.

In some embodiments, the assay comprises contacting the biological sample with a primer or probe, such as an alteration-specific primer or alteration-specific probe, that specifically hybridizes to a NOTCH3 variant genomic sequence, variant mRNA sequence, or variant cDNA sequence and not the corresponding NOTCH3 reference sequence under stringent conditions, and determining whether hybridization has occurred.

In some embodiments, the assay comprises RNA sequencing (RNA-Seq). In some embodiments, the assays also comprise reverse transcribing mRNA into cDNA, such as by the reverse transcriptase polymerase chain reaction (RT-PCR).

- 45 -

In some embodiments, the methods utilize probes and primers of sufficient nucleotide length to bind to the target nucleotide sequence and specifically detect and/or identify a polynucleotide comprising a NOTCH3 variant genomic nucleic acid molecule, variant mRNA molecule, or variant cDNA molecule. The hybridization conditions or reaction conditions can be
5 determined by the operator to achieve this result. The nucleotide length may be any length that is sufficient for use in a detection method of choice, including any assay described or exemplified herein. Such probes and primers can hybridize specifically to a target nucleotide sequence under high stringency hybridization conditions. Probes and primers may have complete nucleotide sequence identity of contiguous nucleotides within the target nucleotide
10 sequence, although probes differing from the target nucleotide sequence and that retain the ability to specifically detect and/or identify a target nucleotide sequence may be designed by conventional methods. Probes and primers can have about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity or complementarity with the nucleotide sequence of the target
15 nucleic acid molecule.

In some embodiments, to determine whether a NOTCH3 nucleic acid molecule (genomic nucleic acid molecule, mRNA molecule, or cDNA molecule), or complement thereof, within a biological sample comprises a nucleotide sequence comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2 (genomic nucleic acid molecule),
20 the biological sample can be subjected to an amplification method using a primer pair that includes a first primer derived from the 5' flanking sequence adjacent to a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, and a second primer derived from the 3' flanking sequence adjacent to a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, to produce an amplicon that is indicative of the presence of the SNP
25 at positions encoding a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2. In some embodiments, the amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. This distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. Optionally,
30 the primer pair flanks a region including positions comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, and at least 1, 2, 3, 4, 5, 6, 7, 8, 9,

- 46 -

10, or more nucleotides on each side of positions comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2.

In some embodiments, to determine whether a NOTCH3 nucleic acid molecule (genomic nucleic acid molecule, mRNA molecule, or cDNA molecule), or complement thereof, within a biological sample comprises a nucleotide sequence comprising a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8 (mRNA molecule), or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18 (cDNA molecule), the biological sample can be subjected to an amplification method using a primer pair that includes a first primer derived from the 5' flanking sequence adjacent to a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18, and a second primer derived from the 3' flanking sequence adjacent to a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18 to produce an amplicon that is indicative of the presence of the SNP at positions encoding a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18. In some embodiments, the amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. This distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. Optionally, the primer pair flanks a region including positions comprising a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18, and at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides on each side of positions comprising a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8, or a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18.

In some embodiments, to determine whether a NOTCH3 nucleic acid molecule (mRNA molecule, or cDNA molecule), or complement thereof, within a biological sample comprises a nucleotide sequence comprising a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9 (mRNA molecule), or a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19 (cDNA molecule), the biological sample can be subjected to an amplification method using a primer pair that includes a first primer derived from the 5'

- 47 -

flanking sequence adjacent to a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19, and a second primer derived from the 3' flanking sequence adjacent to a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or a thymine at a position
5 corresponding to position 3,767 according to SEQ ID NO:19 to produce an amplicon that is indicative of the presence of the SNP at positions encoding a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19. In some embodiments, the amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any
10 length of amplicon producible by a DNA amplification protocol. This distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. Optionally, the primer pair flanks a region including positions comprising a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19, and at least
15 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides on each side of positions comprising a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9, or a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19.

In some embodiments, to determine whether a NOTCH3 nucleic acid molecule (mRNA molecule, or cDNA molecule), or complement thereof, within a biological sample comprises a
20 nucleotide sequence comprising a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10 (mRNA molecule), or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20 (cDNA molecule), the biological sample can be subjected to an amplification method using a primer pair that includes a first primer derived from the 5' flanking sequence adjacent to a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20, and a second primer derived from the 3' flanking sequence adjacent to a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20 to produce an amplicon that is
25 indicative of the presence of the SNP at positions encoding a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20. In some embodiments, the amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any
30

- 48 -

length of amplicon producible by a DNA amplification protocol. This distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. Optionally, the primer pair flanks a region including positions comprising a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or
5 a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20, and at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides on each side of positions comprising a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10, or a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20.

In some embodiments, to determine whether a NOTCH3 nucleic acid molecule (mRNA
10 molecule, or cDNA molecule), or complement thereof, within a biological sample comprises a nucleotide sequence comprising a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11 (mRNA molecule), or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21 (cDNA molecule), the biological sample can be subjected to an amplification method using a primer pair that includes a first primer derived from the 5'
15 flanking sequence adjacent to a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21, and a second primer derived from the 3' flanking sequence adjacent to a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21 to produce an amplicon that is
20 indicative of the presence of the SNP at positions encoding a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21. In some embodiments, the amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. This distance can range from
25 one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. Optionally, the primer pair flanks a region including positions comprising a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11, or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21, and at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides on each side of positions comprising a uracil at a
30 position corresponding to position 3,769 according to SEQ ID NO:11, or a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21.

- 49 -

In some embodiments, to determine whether a NOTCH3 nucleic acid molecule (mRNA molecule, or cDNA molecule), or complement thereof, within a biological sample comprises a nucleotide sequence comprising a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12 (mRNA molecule), or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22 (cDNA molecule), the biological sample can be subjected to an amplification method using a primer pair that includes a first primer derived from the 5' flanking sequence adjacent to a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22, and a second primer derived from the 3' flanking sequence adjacent to a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22 to produce an amplicon that is indicative of the presence of the SNP at positions encoding a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22. In some embodiments, the amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. This distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. Optionally, the primer pair flanks a region including positions comprising thymine at a position corresponding to position a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22, and at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides on each side of positions comprising a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12, or a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22.

Similar amplicons can be generated from the mRNA and/or cDNA sequences. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose, such as the PCR primer analysis tool in Vector NTI version 10 (Informax Inc., Bethesda Md.); PrimerSelect (DNASTAR Inc., Madison, Wis.); and Primer3 (Version 0.4.0.COPYRG.T., 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). Additionally, the sequence can be visually scanned and primers manually identified using known guidelines.

- 50 -

Illustrative examples of nucleic acid sequencing techniques include, but are not limited to, chain terminator (Sanger) sequencing and dye terminator sequencing. Other methods involve nucleic acid hybridization methods other than sequencing, including using labeled primers or probes directed against purified DNA, amplified DNA, and fixed cell preparations (fluorescence in situ hybridization (FISH)). In some methods, a target nucleic acid molecule may be amplified prior to or simultaneous with detection. Illustrative examples of nucleic acid amplification techniques include, but are not limited to, polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), and nucleic acid sequence based amplification (NASBA). Other methods include, but are not limited to, ligase chain reaction, strand displacement amplification, and thermophilic SDA (tSDA).

In hybridization techniques, stringent conditions can be employed such that a probe or primer will specifically hybridize to its target. In some embodiments, a polynucleotide primer or probe under stringent conditions will hybridize to its target sequence to a detectably greater degree than to other non-target sequences, such as, at least 2-fold, at least 3-fold, at least 4-fold, or more over background, including over 10-fold over background. In some embodiments, a polynucleotide primer or probe under stringent conditions will hybridize to its target nucleotide sequence to a detectably greater degree than to other nucleotide sequences by at least 2-fold. In some embodiments, a polynucleotide primer or probe under stringent conditions will hybridize to its target nucleotide sequence to a detectably greater degree than to other nucleotide sequences by at least 3-fold. In some embodiments, a polynucleotide primer or probe under stringent conditions will hybridize to its target nucleotide sequence to a detectably greater degree than to other nucleotide sequences by at least 4-fold. In some embodiments, a polynucleotide primer or probe under stringent conditions will hybridize to its target nucleotide sequence to a detectably greater degree than to other nucleotide sequences by over 10-fold over background. Stringent conditions are sequence-dependent and will be different in different circumstances.

Appropriate stringency conditions which promote DNA hybridization, for example, 6X sodium chloride/sodium citrate (SSC) at about 45°C., followed by a wash of 2X SSC at 50°C, are known or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Typically, stringent conditions for hybridization and detection will be those in which the salt concentration is less than about 1.5 M Na⁺ ion, typically about 0.01 to 1.0 M Na⁺ ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about

- 51 -

30°C for short probes (such as, for example, 10 to 50 nucleotides) and at least about 60°C for longer probes (such as, for example, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours. The duration of the wash time will be at least a length of time sufficient to reach equilibrium.

The present disclosure also provides methods of detecting the presence of a NOTCH3 predicted gain-of-function polypeptide comprising performing an assay on a biological sample obtained from the subject to determine whether a NOTCH3 polypeptide in the subject contains one or more variations that causes the polypeptide to have a gain-of-function (partial or complete) or predicted gain-of-function (partial or complete). The NOTCH3 predicted gain-of-function polypeptide can be any of the NOTCH3 variant polypeptides described herein. In some embodiments, the methods detect the presence of NOTCH3 Arg1178Cys, Arg1231Cys, or Arg1182Cys. In some embodiments, the methods detect the presence of NOTCH3 Arg1178Cys. In some embodiments, the methods detect the presence of NOTCH3 Arg1231Cys. In some embodiments, the methods detect the presence of NOTCH3 Arg1182Cys.

In any of the embodiments described herein, the NOTCH3 variant polypeptides can be any Cys-altering variant (they change from or to a cysteine residue). In some embodiments the Cys-altering variant is located within the 34 EGF-like repeats in the NOTCH3 extracellular domain (defined by Uniprot; see world wide web at "uniprot.org/blast/?about=Q9UM47[1335-1373]&key=Domain)). In any of the embodiments described herein, the NOTCH3 variant polypeptide can be any of the variants listed in Table 1 (ENST00000263388).

In some embodiments, the methods comprise performing an assay on a sample obtained from a subject to determine whether a NOTCH3 polypeptide in the sample comprises a cysteine at a position corresponding to position 1,178 according to SEQ ID NO:26. In some embodiments, the methods comprise performing an assay on a sample obtained from a subject to determine whether a NOTCH3 polypeptide in the sample comprises a cysteine at a position corresponding to position 1,231 according to SEQ ID NO:27. In some embodiments, the methods comprise performing an assay on a sample obtained from a subject to determine whether a NOTCH3 polypeptide in the sample comprises a cysteine at a position corresponding to position 1,182 according to SEQ ID NO:28.

- 52 -

In some embodiments, the detecting step comprises sequencing at least a portion of the polypeptide that comprises a position corresponding to position 1,178 according to SEQ ID NO:26 or SEQ ID NO:23. In some embodiments, the detecting step comprises sequencing at least a portion of the polypeptide that comprises a position corresponding to position 1,231 according to SEQ ID NO:27 or SEQ ID NO:24. In some embodiments, the detecting step comprises sequencing at least a portion of the polypeptide that comprises a position corresponding to position 1,182 according to SEQ ID NO:28 or SEQ ID NO:25.

In some embodiments, the detecting step comprises an immunoassay for detecting the presence of a polypeptide that comprises a position corresponding to position 1,178 according to SEQ ID NO:26 or SEQ ID NO:23. In some embodiments, the detecting step comprises an immunoassay for detecting the presence of a polypeptide that comprises a position corresponding to position 1,231 according to SEQ ID NO:27 or SEQ ID NO:24. In some embodiments, the detecting step comprises an immunoassay for detecting the presence of a polypeptide that comprises a position corresponding to position 1,182 according to SEQ ID NO:28 or SEQ ID NO:25.

In some embodiments, when the subject does not have a NOTCH3 predicted gain-of-function polypeptide, the subject does not have an increased risk for developing a cerebrovascular disease or any of CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke. In some embodiments, when the subject has a NOTCH3 predicted gain-of-function polypeptide, the subject has an increased risk for developing a cerebrovascular disease or any of CADASIL, subcortical stroke, ischemic stroke, hemorrhagic stroke, or parenchymal stroke.

The present disclosure also provides isolated nucleic acid molecules that hybridize to NOTCH3 variant genomic nucleic acid molecules, NOTCH3 variant mRNA molecules, and/or NOTCH3 variant cDNA molecules (such as any of the genomic variant nucleic acid molecules, mRNA variant molecules, and cDNA variant molecules disclosed herein). In some embodiments, the isolated nucleic acid molecules hybridize to a portion of the NOTCH3 nucleic acid molecule that includes a position corresponding to position 21,944 according to SEQ ID NO:2. In some embodiments, the isolated nucleic acid molecules hybridize to a portion of the NOTCH3 nucleic acid molecule that includes a position corresponding to: position 3,781 according to SEQ ID NO:8, or position 3,781 according to SEQ ID NO:18. In some embodiments, the isolated nucleic acid molecules hybridize to a portion of the NOTCH3 nucleic acid molecule that includes a

- 53 -

position corresponding to: position 3,767 according to SEQ ID NO:9, or position 3,767 according to SEQ ID NO:19. In some embodiments, the isolated nucleic acid molecules hybridize to a portion of the NOTCH3 nucleic acid molecule that includes a position corresponding to: position 3,532 according to SEQ ID NO:10, or position 3,532 according to SEQ ID NO:20. In some
5 embodiments, the isolated nucleic acid molecules hybridize to a portion of the NOTCH3 nucleic acid molecule that includes a position corresponding to: position 3,769 according to SEQ ID NO:11, or position 3,769 according to SEQ ID NO:21. In some embodiments, the isolated nucleic acid molecules hybridize to a portion of the NOTCH3 nucleic acid molecule that includes a position corresponding to: position 3,544 according to SEQ ID NO:12, or position 3,544
10 according to SEQ ID NO:22.

 In some embodiments, such isolated nucleic acid molecules comprise or consist of at least about 5, at least about 8, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least about 21, at least about 22, at least about 23, at
15 least about 24, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, at least about 800, at least about 900, at least about 1000,
20 at least about 2000, at least about 3000, at least about 4000, or at least about 5000 nucleotides. In some embodiments, such isolated nucleic acid molecules comprise or consist of at least about 5, at least about 8, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least about 21, at least about 22, at least about 23, at
25 least about 24, or at least about 25 nucleotides. In some embodiments, the isolated nucleic acid molecules comprise or consist of at least about 18 nucleotides. In some embodiments, the isolated nucleic acid molecules comprise or consists of at least about 15 nucleotides. In some embodiments, the isolated nucleic acid molecules consist of or comprise from about 10 to about 35, from about 10 to about 30, from about 10 to about 25, from about 12 to about 30,
30 from about 12 to about 28, from about 12 to about 24, from about 15 to about 30, from about 15 to about 25, from about 18 to about 30, from about 18 to about 25, from about 18 to about 24, or from about 18 to about 22 nucleotides. In some embodiments, the isolated nucleic acid

- 54 -

molecules consist of or comprise from about 18 to about 30 nucleotides. In some embodiments, the isolated nucleic acid molecules comprise or consist of at least about 15 nucleotides to at least about 35 nucleotides.

In some embodiments, such isolated nucleic acid molecules hybridize to NOTCH3
5 variant nucleic acid molecules (such as genomic nucleic acid molecules, mRNA molecules, and/or cDNA molecules) under stringent conditions. Such nucleic acid molecules can be used, for example, as probes, primers, alteration-specific probes, or alteration-specific primers as described or exemplified herein, and include, without limitation primers, probes, antisense RNAs, shRNAs, and siRNAs, each of which is described in more detail elsewhere herein, and can
10 be used in any of the methods described herein.

In some embodiments, the isolated nucleic acid molecules hybridize to at least about 15 contiguous nucleotides of a nucleic acid molecule that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to
15 NOTCH3 variant genomic nucleic acid molecules, NOTCH3 variant mRNA molecules, and/or NOTCH3 variant cDNA molecules. In some embodiments, the isolated nucleic acid molecules consist of or comprise from about 15 to about 100 nucleotides, or from about 15 to about 35 nucleotides. In some embodiments, the isolated nucleic acid molecules consist of or comprise from about 15 to about 100 nucleotides. In some embodiments, the isolated nucleic acid
20 molecules consist of or comprise from about 15 to about 35 nucleotides.

In some embodiments, the isolated alteration-specific probes or alteration-specific primers comprise at least about 15 nucleotides, wherein the alteration-specific probe or alteration-specific primer comprises a nucleotide sequence which is complementary to a portion of a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the portion
25 comprises a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof. In some embodiments, the alteration-specific probe or alteration-specific primer comprises a nucleotide sequence which is complementary to a portion of a nucleotide sequence comprising positions corresponding to positions 21,944-21,946 according to SEQ ID NO:2, or the complement thereof.

30 In some embodiments, the isolated alteration-specific probes or alteration-specific primers comprise at least about 15 nucleotides, wherein the alteration-specific probe or alteration-specific primer comprises a nucleotide sequence which is complementary to a

- 55 -

portion of a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the portion comprises a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; or position 3,781 according to SEQ ID NO:18, or the complement thereof. In some embodiments, the alteration-specific probe or alteration-specific primer comprises a
5 nucleotide sequence which is complementary to a portion of a nucleotide sequence comprising positions corresponding to: positions 3,781-3,783 according to SEQ ID NO:8, or the complement thereof; and/or positions 3,781-3,783 according to SEQ ID NO:18, or the complement thereof.

In some embodiments, the isolated alteration-specific probes or alteration-specific primers comprise at least about 15 nucleotides, wherein the alteration-specific probe or
10 alteration-specific primer comprises a nucleotide sequence which is complementary to a portion of a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the portion comprises a position corresponding to: position 3,767 according to SEQ ID NO:9, or the complement thereof; or position 3,767 according to SEQ ID NO:19, or the complement thereof. In some embodiments, the alteration-specific probe or alteration-specific primer comprises a
15 nucleotide sequence which is complementary to a portion of a nucleotide sequence comprising positions corresponding to: positions 3,767-3,769 according to SEQ ID NO:9, or the complement thereof; and/or positions 3,767-3,769 according to SEQ ID NO:19, or the complement thereof.

In some embodiments, the isolated alteration-specific probes or alteration-specific primers comprise at least about 15 nucleotides, wherein the alteration-specific probe or
20 alteration-specific primer comprises a nucleotide sequence which is complementary to a portion of a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the portion comprises a position corresponding to: position 3,532 according to SEQ ID NO:10, or the complement thereof; or position 3,532 according to SEQ ID NO:20, or the complement thereof. In some embodiments, the alteration-specific probe or alteration-specific primer comprises a
25 nucleotide sequence which is complementary to a portion of a nucleotide sequence comprising positions corresponding to: positions 3,532-3,534 according to SEQ ID NO:10, or the complement thereof; and/or positions 3,532-3,534 according to SEQ ID NO:20, or the complement thereof.

In some embodiments, the isolated alteration-specific probes or alteration-specific
30 primers comprise at least about 15 nucleotides, wherein the alteration-specific probe or alteration-specific primer comprises a nucleotide sequence which is complementary to a portion of a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the portion

- 56 -

comprises a position corresponding to: position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,769 according to SEQ ID NO:21, or the complement thereof. In some embodiments, the alteration-specific probe or alteration-specific primer comprises a nucleotide sequence which is complementary to a portion of a nucleotide sequence comprising

5 positions corresponding to: positions 3,769-3,771 according to SEQ ID NO:11, or the complement thereof; and/or positions 3,769-3,771 according to SEQ ID NO:21, or the complement thereof.

In some embodiments, the isolated alteration-specific probes or alteration-specific primers comprise at least about 15 nucleotides, wherein the alteration-specific probe or

10 alteration-specific primer comprises a nucleotide sequence which is complementary to a portion of a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the portion comprises a position corresponding to: position 3,544 according to SEQ ID NO:12, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof. In some embodiments, the alteration-specific probe or alteration-specific primer comprises a

15 nucleotide sequence which is complementary to a portion of a nucleotide sequence comprising positions corresponding to: positions 3,544-3,546 according to SEQ ID NO:12, or the complement thereof; and/or positions 3,544-3,546 according to SEQ ID NO:22, or the complement thereof.

In some embodiments, the alteration-specific probes and alteration-specific primers

20 comprise DNA. In some embodiments, the alteration-specific probes and alteration-specific primers comprise RNA.

In some embodiments, the probes and primers described herein (including alteration-specific probes and alteration-specific primers) have a nucleotide sequence that specifically hybridizes to any of the nucleic acid molecules disclosed herein, or the complement thereof. In

25 some embodiments, the probes and primers specifically hybridize to any of the nucleic acid molecules disclosed herein under stringent conditions.

In some embodiments, the primers, including alteration-specific primers, can be used in second generation sequencing or high throughput sequencing. In some instances, the primers, including alteration-specific primers, can be modified. In particular, the primers can

30 comprise various modifications that are used at different steps of, for example, Massive Parallel Signature Sequencing (MPSS), Polony sequencing, and 454 Pyrosequencing. Modified primers can be used at several steps of the process, including biotinylated primers in the cloning step

- 57 -

and fluorescently labeled primers used at the bead loading step and detection step. Polony sequencing is generally performed using a paired-end tags library wherein each molecule of DNA template is about 135 bp in length. Biotinylated primers are used at the bead loading step and emulsion PCR. Fluorescently labeled degenerate nonamer oligonucleotides are used at the
5 detection step. An adaptor can contain a 5'-biotin tag for immobilization of the DNA library onto streptavidin-coated beads.

The probes and primers described herein can be used to detect a nucleotide variation within any of the NOTCH3 variant genomic nucleic acid molecules, NOTCH3 variant mRNA molecules, and/or NOTCH3 variant cDNA molecules disclosed herein. The primers described
10 herein can be used to amplify the NOTCH3 variant genomic nucleic acid molecules, NOTCH3 variant mRNA molecules, or NOTCH3 variant cDNA molecules, or a fragment thereof.

The present disclosure also provides pairs of primers comprising any of the primers described above. For example, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 21,944 according to SEQ ID NO:1 (rather than a thymine) in a
15 particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference genomic nucleic acid molecule. Conversely, if one of the primers' 3'-ends hybridizes to a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2 (rather than a cytosine) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3
20 variant genomic nucleic acid molecule. In some embodiments, the nucleotide of the primer complementary to the thymine at a position corresponding to position 21,944 according to SEQ ID NO:2 can be at the 3' end of the primer.

The present disclosure also provides pairs of primers comprising any of the primers described above. For example, if one of the primers' 3'-ends hybridizes to a cytosine at a
25 position corresponding to position 3,781 according to SEQ ID NO:3 (rather than a uracil) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference mRNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a uracil at a position corresponding to position 3,781 according to SEQ ID NO:8 (rather than a cytosine) in a particular NOTCH3 mRNA molecule, then the presence
30 of the amplified fragment would indicate the presence of the NOTCH3 variant mRNA molecule. In some embodiments, the nucleotide of the primer complementary to the uracil at a position corresponding to position 3,781 according to SEQ ID NO:8 can be at the 3' end of the primer. In

- 58 -

addition, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,781 according to SEQ ID NO:13 (rather than a thymine) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference cDNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18 (rather than a cytosine) in a particular NOTCH3 cDNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant cDNA molecule. In some embodiments, the nucleotide of the primer complementary to the uracil at a position corresponding to position 3,781 according to SEQ ID NO:18 can be at the 3' end of the primer.

10 The present disclosure also provides pairs of primers comprising any of the primers described above. For example, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,767 according to SEQ ID NO:4 (rather than a uracil) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference mRNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a uracil at a position corresponding to position 3,767 according to SEQ ID NO:9 (rather than a cytosine) in a particular NOTCH3 mRNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant mRNA molecule. In some embodiments, the nucleotide of the primer complementary to the uracil at a position corresponding to position 3,767 according to SEQ ID NO:9 can be at the 3' end of the primer. In addition, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,767 according to SEQ ID NO:14 (rather than a thymine) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference cDNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a thymine at a position corresponding to position 3,767 according to SEQ ID NO:19 (rather than a cytosine) in a particular NOTCH3 cDNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant cDNA molecule. In some embodiments, the nucleotide of the primer complementary to the thymine at a position corresponding to position 3,767 according to SEQ ID NO:19 can be at the 3' end of the primer.

 The present disclosure also provides pairs of primers comprising any of the primers described above. For example, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,532 according to SEQ ID NO:5 (rather than a uracil) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would

- 59 -

indicate the presence of a NOTCH3 reference mRNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a uracil at a position corresponding to position 3,532 according to SEQ ID NO:10 (rather than a cytosine) in a particular NOTCH3 mRNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant mRNA molecule. In some embodiments, the nucleotide of the primer complementary to the uracil at a position corresponding to position 3,532 according to SEQ ID NO:10 can be at the 3' end of the primer. In addition, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,532 according to SEQ ID NO:15 (rather than a thymine) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference cDNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a thymine at a position corresponding to position 3,532 according to SEQ ID NO:20 (rather than a cytosine) in a particular NOTCH3 cDNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant cDNA molecule. In some embodiments, the nucleotide of the primer complementary to the thymine at a position corresponding to position 3,532 according to SEQ ID NO:20 can be at the 3' end of the primer.

The present disclosure also provides pairs of primers comprising any of the primers described above. For example, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,769 according to SEQ ID NO:6 (rather than a uracil) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference mRNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a uracil at a position corresponding to position 3,769 according to SEQ ID NO:11 (rather than a cytosine) in a particular NOTCH3 mRNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant mRNA molecule. In some embodiments, the nucleotide of the primer complementary to the uracil at a position corresponding to position 3,769 according to SEQ ID NO:11 can be at the 3' end of the primer. In addition, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,769 according to SEQ ID NO:16 (rather than a thymine) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference cDNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a thymine at a position corresponding to position 3,769 according to SEQ ID NO:21 (rather than a cytosine) in a particular NOTCH3 cDNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant cDNA molecule. In

- 60 -

some embodiments, the nucleotide of the primer complementary to the thymine at a position corresponding to position 3,769 according to SEQ ID NO:21 can be at the 3' end of the primer.

The present disclosure also provides pairs of primers comprising any of the primers described above. For example, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,544 according to SEQ ID NO:7 (rather than a uracil) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference mRNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a uracil at a position corresponding to position 3,544 according to SEQ ID NO:12 (rather than a cytosine) in a particular NOTCH3 mRNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant mRNA molecule. In some embodiments, the nucleotide of the primer complementary to the uracil at a position corresponding to position 3,544 according to SEQ ID NO:12 can be at the 3' end of the primer. In addition, if one of the primers' 3'-ends hybridizes to a cytosine at a position corresponding to position 3,544 according to SEQ ID NO:17 (rather than a thymine) in a particular NOTCH3 nucleic acid molecule, then the presence of the amplified fragment would indicate the presence of a NOTCH3 reference cDNA molecule. Conversely, if one of the primers' 3'-ends hybridizes to a thymine at a position corresponding to position 3,544 according to SEQ ID NO:22 (rather than a cytosine) in a particular NOTCH3 cDNA molecule, then the presence of the amplified fragment would indicate the presence of the NOTCH3 variant cDNA molecule. In some embodiments, the nucleotide of the primer complementary to the thymine at a position corresponding to position 3,544 according to SEQ ID NO:22 can be at the 3' end of the primer.

In the context of the present disclosure "specifically hybridizes" means that the probe or primer (such as, for example, the alteration-specific probe or alteration-specific primer) does not hybridize to a nucleic acid sequence encoding a NOTCH3 reference genomic nucleic acid molecule, a NOTCH3 reference mRNA molecule, and/or a NOTCH3 reference cDNA molecule.

In some embodiments, the probes (such as, for example, an alteration-specific probe) comprise a label. In some embodiments, the label is a fluorescent label, a radiolabel, or biotin.

The present disclosure also provides supports comprising a substrate to which any one or more of the probes disclosed herein is attached. Solid supports are solid-state substrates or supports with which molecules, such as any of the probes disclosed herein, can be associated. A form of solid support is an array. Another form of solid support is an array detector. An array detector is a solid support to which multiple different probes have been coupled in an array,

- 61 -

grid, or other organized pattern. A form for a solid-state substrate is a microtiter dish, such as a standard 96-well type. In some embodiments, a multiwell glass slide can be employed that normally contains one array per well.

The nucleotide sequence of a NOTCH3 reference genomic nucleic acid molecule is set
5 forth in SEQ ID NO:1. Referring to SEQ ID NO:1, position 21,944 is a cytosine.

A variant genomic nucleic acid molecule of NOTCH3 exists, wherein the cytosine at position 21,944 is replaced with a thymine. The nucleotide sequence of this NOTCH3 variant genomic nucleic acid molecule is set forth in SEQ ID NO:2.

The nucleotide sequence of a NOTCH3 reference mRNA molecule is set forth in SEQ ID
10 NO:3. Referring to SEQ ID NO:3, position 3,781 is a cytosine.

The nucleotide sequence of another NOTCH3 reference mRNA molecule is set forth in SEQ ID NO:4. Referring to SEQ ID NO:4, position 3,767 is a cytosine.

The nucleotide sequence of another NOTCH3 reference mRNA molecule is set forth in SEQ ID NO:5. Referring to SEQ ID NO:5, position 3,532 is a cytosine.

15 The nucleotide sequence of another NOTCH3 reference mRNA molecule is set forth in SEQ ID NO:6. Referring to SEQ ID NO:6, position 3,769 is a cytosine.

The nucleotide sequence of another NOTCH3 reference mRNA molecule is set forth in SEQ ID NO:7. Referring to SEQ ID NO:7, position 3,544 is a cytosine.

A variant mRNA molecule of NOTCH3 exists, wherein the cytosine at position 3,781 is
20 replaced with a uracil. The nucleotide sequence of this NOTCH3 variant mRNA molecule is set forth in SEQ ID NO:8.

Another variant mRNA molecule of NOTCH3 exists, wherein the cytosine at position 3,767 is replaced with a uracil. The nucleotide sequence of this NOTCH3 variant mRNA molecule is set forth in SEQ ID NO:9.

25 Another variant mRNA molecule of NOTCH3 exists, wherein the cytosine at position 3,532 is replaced with a uracil. The nucleotide sequence of this NOTCH3 variant mRNA molecule is set forth in SEQ ID NO:10.

Another variant mRNA molecule of NOTCH3 exists, wherein the cytosine at position 3,769 is replaced with a uracil. The nucleotide sequence of this NOTCH3 variant mRNA
30 molecule is set forth in SEQ ID NO:11.

- 62 -

Another variant mRNA molecule of NOTCH3 exists, wherein the cytosine at position 3,544 is replaced with a uracil. The nucleotide sequence of this NOTCH3 variant mRNA molecule is set forth in SEQ ID NO:12.

The nucleotide sequence of a NOTCH3 reference cDNA molecule is set forth in SEQ ID NO:13. Referring to SEQ ID NO:13, position 3,781 is a cytosine.

The nucleotide sequence of another NOTCH3 reference cDNA molecule is set forth in SEQ ID NO:14. Referring to SEQ ID NO:14, position 3,767 is a cytosine.

The nucleotide sequence of another NOTCH3 reference cDNA molecule is set forth in SEQ ID NO:15. Referring to SEQ ID NO:15, position 3,532 is a cytosine.

The nucleotide sequence of another NOTCH3 reference cDNA molecule is set forth in SEQ ID NO:16. Referring to SEQ ID NO:16, position 3,769 is a cytosine.

The nucleotide sequence of another NOTCH3 reference cDNA molecule is set forth in SEQ ID NO:17. Referring to SEQ ID NO:17, position 3,544 is a cytosine.

A variant cDNA molecule of NOTCH3 exists, wherein the cytosine at position 3,781 is replaced with a thymine. The nucleotide sequence of this NOTCH3 variant cDNA molecule is set forth in SEQ ID NO:18.

Another variant cDNA molecule of NOTCH3 exists, wherein the cytosine at position 3,767 is replaced with a thymine. The nucleotide sequence of this NOTCH3 variant cDNA molecule is set forth in SEQ ID NO:19.

Another variant cDNA molecule of NOTCH3 exists, wherein the cytosine at position 3,532 is replaced with a thymine. The nucleotide sequence of this NOTCH3 variant cDNA molecule is set forth in SEQ ID NO:20.

Another variant cDNA molecule of NOTCH3 exists, wherein the cytosine at position 3,769 is replaced with a thymine. The nucleotide sequence of this NOTCH3 variant cDNA molecule is set forth in SEQ ID NO:21.

Another variant cDNA molecule of NOTCH3 exists, wherein the cytosine at position 3,544 is replaced with a thymine. The nucleotide sequence of this NOTCH3 variant cDNA molecule is set forth in SEQ ID NO:22.

The genomic nucleic acid molecules, mRNA molecules, and cDNA molecules can be from any organism. For example, the genomic nucleic acid molecules, mRNA molecules, and cDNA molecules can be human or an ortholog from another organism, such as a non-human mammal, a rodent, a mouse, or a rat. It is understood that gene sequences within a population

- 63 -

can vary due to polymorphisms such as single-nucleotide polymorphisms. The examples provided herein are only exemplary sequences. Other sequences are also possible.

Also provided herein are functional polynucleotides that can interact with the disclosed nucleic acid molecules. Examples of functional polynucleotides include, but are not
5 limited to, antisense molecules, aptamers, ribozymes, triplex forming molecules, and external guide sequences. The functional polynucleotides can act as effectors, inhibitors, modulators, and stimulators of a specific activity possessed by a target molecule, or the functional polynucleotides can possess a *de novo* activity independent of any other molecules.

The isolated nucleic acid molecules disclosed herein can comprise RNA, DNA, or both
10 RNA and DNA. The isolated nucleic acid molecules can also be linked or fused to a heterologous nucleic acid sequence, such as in a vector, or a heterologous label. For example, the isolated nucleic acid molecules disclosed herein can be within a vector or as an exogenous donor sequence comprising the isolated nucleic acid molecule and a heterologous nucleic acid sequence. The isolated nucleic acid molecules can also be linked or fused to a heterologous
15 label. The label can be directly detectable (such as, for example, fluorophore) or indirectly detectable (such as, for example, hapten, enzyme, or fluorophore quencher). Such labels can be detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Such labels include, for example, radiolabels, pigments, dyes, chromogens, spin labels, and fluorescent labels. The label can also be, for example, a chemiluminescent substance; a
20 metal-containing substance; or an enzyme, where there occurs an enzyme-dependent secondary generation of signal. The term "label" can also refer to a "tag" or hapten that can bind selectively to a conjugated molecule such that the conjugated molecule, when added subsequently along with a substrate, is used to generate a detectable signal. For example, biotin can be used as a tag along with an avidin or streptavidin conjugate of horseradish
25 peroxidase (HRP) to bind to the tag, and examined using a calorimetric substrate (such as, for example, tetramethylbenzidine (TMB)) or a fluorogenic substrate to detect the presence of HRP. Exemplary labels that can be used as tags to facilitate purification include, but are not limited to, myc, HA, FLAG or 3XFLAG, 6XHis or polyhistidine, glutathione-S-transferase (GST), maltose binding protein, an epitope tag, or the Fc portion of immunoglobulin. Numerous labels
30 include, for example, particles, fluorophores, haptens, enzymes and their calorimetric, fluorogenic and chemiluminescent substrates and other labels.

- 64 -

The disclosed nucleic acid molecules can comprise, for example, nucleotides or non-natural or modified nucleotides, such as nucleotide analogs or nucleotide substitutes. Such nucleotides include a nucleotide that contains a modified base, sugar, or phosphate group, or that incorporates a non-natural moiety in its structure. Examples of non-natural nucleotides include, but are not limited to, dideoxynucleotides, biotinylated, aminated, deaminated, alkylated, benzylated, and fluorophor-labeled nucleotides.

The nucleic acid molecules disclosed herein can also comprise one or more nucleotide analogs or substitutions. A nucleotide analog is a nucleotide which contains a modification to either the base, sugar, or phosphate moieties. Modifications to the base moiety include, but are not limited to, natural and synthetic modifications of A, C, G, and T/U, as well as different purine or pyrimidine bases such as, for example, pseudouridine, uracil-5-yl, hypoxanthin-9-yl (I), and 2-aminoadenin-9-yl. Modified bases include, but are not limited to, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo (such as, for example, 5-bromo), 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 7-methyladenine, 8-azaguanine, 8-azaadenine, 7-deazaguanine, 7-deazaadenine, 3-deazaguanine, and 3-deazaadenine.

Nucleotide analogs can also include modifications of the sugar moiety. Modifications to the sugar moiety include, but are not limited to, natural modifications of the ribose and deoxy ribose as well as synthetic modifications. Sugar modifications include, but are not limited to, the following modifications at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl, and alkynyl may be substituted or unsubstituted C₁₋₁₀alkyl or C₂₋₁₀alkenyl, and C₂₋₁₀alkynyl. Exemplary 2' sugar modifications also include, but are not limited to, -O[(CH₂)_nO]_mCH₃, -O(CH₂)_nOCH₃, -O(CH₂)_nNH₂, -O(CH₂)_nCH₃, -O(CH₂)_n-ONH₂, and -O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m, independently, are from 1 to about 10. Other modifications at the 2' position include, but are not limited to, C₁₋₁₀alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an

- 65 -

intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Similar modifications may also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or
5 in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Modified sugars can also include those that contain modifications at the bridging ring oxygen, such as CH₂ and S. Nucleotide sugar analogs can also have sugar mimetics, such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Nucleotide analogs can also be modified at the phosphate moiety. Modified phosphate
10 moieties include, but are not limited to, those that can be modified so that the linkage between two nucleotides contains a phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, methyl and other alkyl phosphonates including 3'-alkylene phosphonate and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates,
15 thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates. These phosphate or modified phosphate linkage between two nucleotides can be through a 3'-5' linkage or a 2'-5' linkage, and the linkage can contain inverted polarity such as 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts, and free acid forms are also included. Nucleotide substitutes also include peptide nucleic acids (PNAs).

20 The present disclosure also provides vectors comprising any one or more of the nucleic acid molecules disclosed herein. In some embodiments, the vectors comprise any one or more of the nucleic acid molecules disclosed herein and a heterologous nucleic acid. The vectors can be viral or nonviral vectors capable of transporting a nucleic acid molecule. In some embodiments, the vector is a plasmid or cosmid (such as, for example, a circular double-
25 stranded DNA into which additional DNA segments can be ligated). In some embodiments, the vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Expression vectors include, but are not limited to, plasmids, cosmids, retroviruses, adenoviruses, adeno-associated viruses (AAV), plant viruses such as cauliflower mosaic virus and tobacco mosaic virus, yeast artificial chromosomes (YACs), Epstein-Barr (EBV)-derived
30 episomes, and other expression vectors known in the art.

Desired regulatory sequences for mammalian host cell expression can include, for example, viral elements that direct high levels of polypeptide expression in mammalian cells,

- 66 -

such as promoters and/or enhancers derived from retroviral LTRs, cytomegalovirus (CMV) (such as, for example, CMV promoter/enhancer), Simian Virus 40 (SV40) (such as, for example, SV40 promoter/enhancer), adenovirus, (such as, for example, the adenovirus major late promoter (AdMLP)), polyoma and strong mammalian promoters such as native immunoglobulin and actin
5 promoters. Methods of expressing polypeptides in bacterial cells or fungal cells (such as, for example, yeast cells) are also well known. A promoter can be, for example, a constitutively active promoter, a conditional promoter, an inducible promoter, a temporally restricted promoter (such as, for example, a developmentally regulated promoter), or a spatially restricted promoter (such as, for example, a cell-specific or tissue-specific promoter).

10 Percent identity (or percent complementarity) between particular stretches of nucleotide sequences within nucleic acid molecules or amino acid sequences within polypeptides can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656) or by using the Gap program (Wisconsin Sequence
15 Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wis.), using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489). Herein, if reference is made to percent sequence identity, the higher percentages of sequence identity are preferred over the lower ones.

The present disclosure also provides compositions comprising any one or more of the
20 isolated nucleic acid molecules, genomic nucleic acid molecules, mRNA molecules, and/or cDNA molecules disclosed herein. In some embodiments, the composition is a pharmaceutical composition. In some embodiments, the compositions comprise a carrier and/or excipient. Examples of carriers include, but are not limited to, poly(lactic acid) (PLA) microspheres, poly(D,L-lactic-coglycolic-acid) (PLGA) microspheres, liposomes, micelles, inverse micelles, lipid
25 cochleates, and lipid microtubules. A carrier may comprise a buffered salt solution such as PBS, HBSS, etc.

As used herein, the phrase "corresponding to" or grammatical variations thereof when used in the context of the numbering of a particular nucleotide or nucleotide sequence or position refers to the numbering of a specified reference sequence when the particular
30 nucleotide or nucleotide sequence is compared to a reference sequence (such as, for example, SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:13). In other words, the residue (such as, for example, nucleotide or amino acid) number or residue (such as, for example, nucleotide or amino acid)

- 67 -

position of a particular polymer is designated with respect to the reference sequence rather than by the actual numerical position of the residue within the particular nucleotide or nucleotide sequence. For example, a particular nucleotide sequence can be aligned to a reference sequence by introducing gaps to optimize residue matches between the two
5 sequences. In these cases, although the gaps are present, the numbering of the residue in the particular nucleotide or nucleotide sequence is made with respect to the reference sequence to which it has been aligned.

For example, a nucleic acid molecule comprising a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position
10 corresponding to position 21,944 according to SEQ ID NO:2 means that if the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule is aligned to the sequence of SEQ ID NO:2, the NOTCH3 sequence has a thymine residue at the position that corresponds to position 21,944 of SEQ ID NO:2. The same applies for mRNA molecules comprising a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a uracil
15 at a position corresponding to position 3,781 according to SEQ ID NO:8, and cDNA molecules comprising a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to position 3,781 according to SEQ ID NO:18. In other words, these phrases refer to a nucleic acid molecule encoding a NOTCH3 polypeptide, wherein the genomic nucleic acid molecule has a nucleotide sequence that
20 comprises a thymine residue that is homologous to the thymine residue at position 21,944 of SEQ ID NO:2 (or wherein the mRNA molecule has a nucleotide sequence that comprises a uracil residue that is homologous to the uracil residue at position 3,781 of SEQ ID NO:8, or wherein the cDNA molecule has a nucleotide sequence that comprises a thymine residue that is homologous to the uracil residue at position 3,781 of SEQ ID NO:18).

25 As described herein, a position within a NOTCH3 genomic nucleic acid molecule that corresponds to position 21,944 according to SEQ ID NO:2, for example, can be identified by performing a sequence alignment between the nucleotide sequence of a particular NOTCH3 nucleic acid molecule and the nucleotide sequence of SEQ ID NO:2. A variety of computational algorithms exist that can be used for performing a sequence alignment to identify a nucleotide
30 position that corresponds to, for example, position 21,944 in SEQ ID NO:2. For example, by using the NCBI BLAST algorithm (Altschul et al., Nucleic Acids Res., 1997, 25, 3389-3402) or

- 68 -

CLUSTALW software (Sievers and Higgins, Methods Mol. Biol., 2014, 1079, 105-116) sequence alignments may be performed. However, sequences can also be aligned manually.

The amino acid sequence of a NOTCH3 reference polypeptide is set forth in SEQ ID NO:23. Referring to SEQ ID NO:23, the NOTCH3 reference polypeptide is 1,286 amino acids in
5 length. Referring to SEQ ID NO:23, position 1,178 is an arginine.

The amino acid sequence of another NOTCH3 reference polypeptide is set forth in SEQ ID NO:24. Referring to SEQ ID NO:24, the NOTCH3 reference polypeptide is 2,321 amino acids in length. Referring to SEQ ID NO:24, position 1,231 is an arginine.

The amino acid sequence of another NOTCH3 reference polypeptide is set forth in SEQ
10 ID NO:25. Referring to SEQ ID NO:25, the NOTCH3 reference polypeptide is 1,289 amino acids in length. Referring to SEQ ID NO:25, position 1,182 is an arginine.

A variant polypeptide of NOTCH3 exists (Arg1178Cys), the amino acid sequence of which is set forth in SEQ ID NO:26. Referring to SEQ ID NO:26, the NOTCH3 variant polypeptide is 1,286 amino acids in length. Referring to SEQ ID NO:26, position 1,178 is a cysteine.

15 Another variant polypeptide of NOTCH3 exists (Arg1231Cys), the amino acid sequence of which is set forth in SEQ ID NO:27. Referring to SEQ ID NO:27, the NOTCH3 variant polypeptide is 2,321 amino acids in length. Referring to SEQ ID NO:27, position 1,231 is a cysteine.

20 Another variant polypeptide of NOTCH3 exists (Arg1182Cys), the amino acid sequence of which is set forth in SEQ ID NO:28. Referring to SEQ ID NO:28, the NOTCH3 variant polypeptide is 1,289 amino acids in length. Referring to SEQ ID NO:28, position 1,182 is a cysteine.

The nucleotide and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter code for
25 amino acids. The nucleotide sequences follow the standard convention of beginning at the 5' end of the sequence and proceeding forward (i.e., from left to right in each line) to the 3' end. Only one strand of each nucleotide sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand. The amino acid sequence follows the standard convention of beginning at the amino terminus of the sequence and
30 proceeding forward (i.e., from left to right in each line) to the carboxy terminus.

The present disclosure also provides therapeutic agents that treat or inhibit a cerebrovascular disease for use in the treatment of a cerebrovascular disease (or for use in the

- 69 -

preparation of a medicament for treating a cerebrovascular disease) in a subject, wherein the subject has any of the NOTCH3 variant genomic nucleic acid molecules, variant mRNA molecules, and/or variant cDNA molecules encoding a NOTCH3 polypeptide described herein. The therapeutic agents that treat or inhibit a cerebrovascular disease can be any of the

5 therapeutic agents that treat or inhibit a cerebrovascular disease described herein.

In some embodiments, the subject is identified as having a genomic nucleic acid molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof.

10 In some embodiments, the subject is identified as having an mRNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according

15 to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof.

In some embodiments, the subject is identified as having a cDNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID

20 NO:22, or the complement thereof.

The present disclosure also provides NOTCH3 agents for use in the treatment of a

25 cerebrovascular disease (or for use in the preparation of a medicament for treating a cerebrovascular disease) in a subject, wherein the subject has any of the NOTCH3 variant genomic nucleic acid molecules, variant mRNA molecules, and/or variant cDNA molecules encoding a NOTCH3 polypeptide described herein. The NOTCH3 agents can be any of the NOTCH3 agents described herein.

30 In some embodiments, the subject is identified as having a genomic nucleic acid molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the

- 70 -

nucleotide sequence comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof.

In some embodiments, the subject is identified as having an mRNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence
5 comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof.

10 In some embodiments, the subject is identified as having a cDNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769
15 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof.

All patent documents, websites, other publications, accession numbers and the like cited above or below are incorporated by reference in their entirety for all purposes to the same extent as if each individual item were specifically and individually indicated to be so
20 incorporated by reference. If different versions of a sequence are associated with an accession number at different times, the version associated with the accession number at the effective filing date of this application is meant. The effective filing date means the earlier of the actual filing date or filing date of a priority application referring to the accession number if applicable. Likewise, if different versions of a publication, website or the like are published at different
25 times, the version most recently published at the effective filing date of the application is meant unless otherwise indicated. Any feature, step, element, embodiment, or aspect of the present disclosure can be used in combination with any other feature, step, element, embodiment, or aspect unless specifically indicated otherwise. Although the present disclosure has been described in some detail by way of illustration and example for purposes of clarity and
30 understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

- 71 -

The following examples are provided to describe the embodiments in greater detail. They are intended to illustrate, not to limit, the claimed embodiments. The following examples provide those of ordinary skill in the art with a disclosure and description of how the compounds, compositions, articles, devices and/or methods described herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the scope of any claims. Efforts have been made to ensure accuracy with respect to numbers (such as, for example, amounts, temperature, etc.), but some errors and deviations may be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

Examples

Example 1: Association of NOTCH3 With an Increased Risk of Developing a Cerebrovascular Disease

Over 19K Pakistanis from 22 clinical sites in 5 cities were recruited for genetic studies, including 4,882 stroke cases and 6,904 controls classified using TOAST and Oxfordshire criteria. The demographics of stroke cases and controls are shown in Table 3 and Table 4.

Table 3: Demographics of stroke cases and controls

Variable	Controls	Cases
Sex (female)	43%	45%
Hypertension	35%	62%
Myocardial infarction	0%	4%
Angina	1%	6%
Diabetes mellitus	18%	25%
Tuberculosis	1%	2%
Family history diabetes mellitus	38%	63%
Family history hypertension	38%	64%
Family history stroke	0%	14%
Family history sudden death	30%	65%
Chronic liver disease	1%	1%
Family history angina	33%	64%

- 72 -

Valvular disease	0%	1%
Batch 2	51%	65%

Table 4: Demographics of stroke cases and controls (continued)

	Controls			Cases		
Variable	Median	SD	Non-missing	Median	SD	Non-missing
Age	55.0	10.1	100%	60.0	13.0	100%
Hypertension age	50.0	8.1	32%	50.0	10.4	53%
Cholesterol mg/dl	183.0	49.3	67%	175.0	57.6	45%
TG mg/dl	171.0	132.8	67%	122.1	86.1	45%
Glucose mg/dl	105.0	73.1	65%	123.0	66.2	44%
HDL mg/dl	36.0	11.4	67%	37.3	12.5	45%
LDL mg/dl	107.4	40.0	66%	109.6	46.6	45%
Creatinine	0.8	0.9	53%	0.9	1.0	42%
VLDL	35.0	28.3	24%	NA	NA	0%
BMI	26.7	4.5	27%	21.2	5.0	5%

Exome sequencing and ExWAS was conducted for 12 binary stroke traits, and
5 identified 36 hits (p-value < 1e-7), including 29 enriched in Pakistan relative to Europeans, of which 7 were missense or LoF (Table 5).

Table 5: Binary stroke traits analyzed in GWAS

Phenotype	N Cases	Tests	Hits	Drifted Hits	Missense or LoF	Manual QC
Stroke	4,882	1,376,990	24	21	7	0
Family history stroke	691	1,530,274	0	2	0	0
Cardioembolism (CE)	413	1,107,635	0	0	0	0
CE probable	387	1,105,914	0	0	0	0
LAA	388	1,104,571	0	0	0	0
LAA probable	366	1,102,944	0	0	0	0

- 73 -

SAA	330	1,098,649	0	0	0	0
SAA probable	291	1,096,023	0	0	0	0
Hemorrhagic	1,683	1,191,892	3	2	0	0
Subcortical	1,416	1,174,008	1	1	1	1
Parenchymal	142	1,086,417	0	0	0	0
Ischemic	2,668	1,249,378	8	5	1	0
IS vs HS	1,683	875,142	0	0	0	0
LACI	386	1,104,571	0	0	0	0
PACI	1,268	1,181,322	0	0	0	0
POCI	481	1,129,686	0	0	0	0
TACI	453	1,127,104	0	0	0	0

The read stack QC excluded 7, pairs of adjacent associated variants (PTPRD: 10 bp, ZNF33A: 6 bp, PTPRB: 3 bp, MYCBP2: 5 bp). One variant was excluded due to MAC=5 and irrelevant biology (increased fertility) (GPR149 3:154429224:A:C, p.Phe131Cys, p-value 7.96E-08, effect 28.04, cases 2698|2693|5|0 controls 6871|6871|0|0, MAF 2.6E-04).

A missense SNV in NOTCH3 (p.Arg1231Cys, GRCh38:Chr19:15179052:G:A, rs201680145) was the only significant hit in CNCD F2 subcortical stroke EXWAS (p-value 2.1e-8, effect size 2.97 (2.01,4.35), MAF 7.1e-3 (Table 6). The variant has elevated MAF in cases, was predicted pathogenic by PolyPhen (score 0.83), and affects an epidermal growth factor (EGF) repeat in a gene highly conserved with mice (93%). The variant adds a Cys in number 31 of 34 EGF repeats in the NOTCH3 extra-cellular domain (ECD), each of which normally has 6 Cys residues. Variants that add or remove a Cys (Cys-altering) from the NOTCH3 ECD are known to be pathogenic in CADASIL.

Table 6: Summary of CNCD F2 subcortical stroke EXWAS results

Gene	NOTCH3
Genome	GRCh38:19:15179052:G:A
DbSNP	rs201680145
Transcript	c.3691C>T
Protein	p.Arg1231Cys

- 74 -

Impact	missense
P value	2.18E-08
Effect	2.97
LCI 95%	2.03
UCI 95%	4.35
Cases RR RA AA	1414 1370 44 0
Controls RR RA AA	6871 6799 71 1
MAF Pakistan	7.10E-03
MAF India	4.20E-03
MAF UK Europeans	1.90E-04

P.Arg1231cys is the only variant associated in the NOTCH3 locus (Figure 1). The variant has elevated MAF in cases, suggesting a risk-increasing effect. There was 1 homozygote, a control. This individual's age was 45, which is young relative to the average age of stroke onset in Pakistan (50), and younger than the median age of subcortical stroke cases (same as age of recruitment in this cohort, 56). We hypothesize that, while this individual may be asymptomatic for stroke, he may exhibit early symptoms of CADASIL such as migraines, GOM accumulation in the vasculature, and white matter hyperintensity and may be at increased risk for stroke. Prior studies suggest more severe CADASIL phenotypes in homozygotes than heterozygotes for this variant.

Table 7: occurrence of cases and controls in the EXWAS grouped by the presence of rs201680145

	Count					
Group	N	Ref/Ref	Ref/Alt	Alt/Alt	Alt alleles	Total alleles
Cases	1414	1370	44	0	44	2828
Controls	6871	6799	71	1	73	13742

	Frequency			
Group	MAF	Ref/Alt	Alt/alt	Ref/Ref
Cases	0.0156	0.0311	0.0000	0.9689

Controls	0.0053	0.0103	0.0001	0.9895
----------	--------	--------	--------	--------

PheWAS of NOTCH3 p.Arg1231Cys revealed nominal p-values for multiple stroke phenotypes, including lacunar artifacts (LACI), small artery atherosclerosis (SAA), partial anterior circulation infarcts (PACI), family history of stroke, and ischemic stroke (Figure 3 and Figure 4).

The NOTCH3 Arg1231Cys variant was observed in RGC cohorts of African, European, Admixed American, and South Asian ancestry. The highest prevalence was observed in South Asians (Table 8). One homozygote was observed in the Admixed Americans (Mexico City cohort), male age 55 with blood pressure 80/116, BMI 25, HbA1c 5.2, smoker since age 18, no history of major CNS bleeding or other disease. Similar to the homozygote in CNCD F2, we hypothesize this individual may have other symptoms of CADASIL not collected for this cohort, such as migraines, GOM, vascular pathology, and brain white matter loss.

Table 8: NOTCH3 Arg1231Cys in other RGC datasets

Data set	African	Admixed American	European	South Asian
Carrier frequency (AA + RA)	1 in 2,618	1 in 1,475	1 in 2,066	1 in 92
AAF	0.000191	0.000339	0.000242	0.005426
HET-RA	24	78	377	420
HOM-AA	0	1	0	6
N	62,867	117,904	777,682	39,806

The Arginine (R) residue is highly conserved across mammalian species, and the p.Arg1231Cys variant is predicted deleterious (PolyPhen2 score 0.843).

Various modifications of the described subject matter, in addition to those described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Each reference (including, but not limited to, journal articles, U.S. and non-U.S. patents, patent application publications, international patent application publications, gene bank accession numbers, and the like) cited in the present application is incorporated herein by reference in its entirety and for all purposes.

- 76 -

What is Claimed is:

1. A method of treating a subject having a cerebrovascular disease, the method comprising administering a Neurogenic Locus Notch Homolog Protein 3 (NOTCH3) agent to the subject.
- 5 2. A method of treating a subject having a subcortical stroke, the method comprising administering a NOTCH3 agent to the subject.
3. A method of treating a subject having an ischemic stroke, the method comprising administering a NOTCH3 agent to the subject.
4. A method of treating a subject having a hemorrhagic stroke, the method comprising
10 administering a NOTCH3 agent to the subject.
5. A method of treating a subject having a parenchymal stroke, the method comprising administering a NOTCH3 agent to the subject.
6. A method of treating a subject having cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), the method comprising administering
15 a NOTCH3 agent to the subject.
7. The method according to any one of claims 1 to 6, wherein the NOTCH3 agent comprises an inhibitory nucleic acid molecule.
8. The method according to claim 7, wherein the inhibitory nucleic acid molecule comprises an antisense nucleic acid molecule, a small interfering RNA (siRNA), or a short hairpin
20 RNA (shRNA) that hybridizes to a NOTCH3 nucleic acid molecule.
9. The method according to any one of claims 1 to 6, wherein the NOTCH3 agent comprises a Cas protein and guide RNA (gRNA) that hybridizes to a gRNA recognition sequence within a NOTCH3 genomic nucleic acid molecule.
10. The method according to claim 9, wherein the Cas protein is Cas9 or Cpf1.
- 25 11. The method according to claim 9 or claim 10, wherein the gRNA recognition sequence includes or is proximate to a position corresponding to position 21,944 according to SEQ ID NO:2.
12. The method according to claim 9 or claim 10, wherein the gRNA recognition sequence is located about 1000, about 500, about 400, about 300, about 200, about 100, about 50, about
30 45, about 40, about 35, about 30, about 25, about 20, about 15, about 10, or about 5 nucleotides from a position corresponding to position 21,944 according to SEQ ID NO:2.

- 77 -

13. The method according to claim 9 or claim 10, wherein a Protospacer Adjacent Motif (PAM) sequence is located from about 2 to about 6 nucleotides downstream of the gRNA recognition sequence.
14. The method according to any one of claims 9 to 13, wherein the gRNA comprises from
5 about 17 nucleotides to about 23 nucleotides.
15. The method according to any one of claims 9 to 13, wherein the gRNA recognition sequence comprises a nucleotide sequence according to any one of SEQ ID NOs:29-43.
16. The method according to any one of claims 1 to 15, further comprising detecting the presence or absence of a NOTCH3 missense variant nucleic acid molecule encoding a NOTCH3
10 predicted gain-of-function polypeptide in a biological sample obtained from the subject.
17. The method according to claim 16, wherein the NOTCH3 missense variant nucleic acid molecule encodes NOTCH3 Arg1178Cys, Arg1231Cys, or Arg1182Cys.
18. The method according to claim 16, wherein the NOTCH3 missense variant nucleic acid molecule encodes NOTCH3 Arg1231Cys.
- 15 19. The method according to claim 17, wherein the NOTCH3 missense variant nucleic acid molecule is:
- a genomic nucleic acid molecule having a nucleotide sequence comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof;
- 20 an mRNA molecule having a nucleotide sequence comprising a uracil at a position corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID NO:12, or the complement thereof;
- 25 or
- a cDNA molecule produced from an mRNA molecule, wherein the cDNA molecule has a nucleotide sequence comprising a thymine at a position corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof; position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or
30 position 3544 according to SEQ ID NO:22, or the complement thereof.

- 78 -

20. The method according to any one of claims 16 to 19, wherein the detecting step is carried out *in vitro*.

21. The method according to any one of claims 16 to 20, wherein the detecting step comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 genomic
5 nucleic acid molecule, or the complement thereof, in the biological sample, wherein the sequenced portion comprises a position corresponding to: position 21,944 according to SEQ ID NO:2, or the complement thereof;

wherein when the sequenced portion of the NOTCH3 genomic nucleic acid molecule in the biological sample comprises a thymine at a position corresponding to position 21,944
10 according to SEQ ID NO:2, or the complement thereof, then the NOTCH3 genomic nucleic acid molecule in the biological sample is a NOTCH3 missense variant genomic nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

22. The method according to any one of claims 16 to 20, wherein the detecting step comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA
15 molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID NO:12, or the complement thereof;

20 wherein when the sequenced portion of the NOTCH3 mRNA molecule in the biological sample comprises a uracil at a position corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID
25 NO:12, or the complement thereof; then the NOTCH3 mRNA molecule in the biological sample is a NOTCH3 missense variant mRNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

23. The method according to any one of claims 16 to 20, wherein the detecting step comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 cDNA
30 molecule produced from an mRNA molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof;

- 79 -

position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or position 3544 according to SEQ ID NO:22, or the complement thereof;

wherein when the sequenced portion of the NOTCH3 cDNA molecule in the biological sample comprises a thymine at a position corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof; position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or position 3544 according to SEQ ID NO:22, or the complement thereof; then the NOTCH3 cDNA molecule produced from an mRNA molecule in the biological sample is a NOTCH3 missense variant cDNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

24. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule, or the complement thereof, that is proximate to a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof;

b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule, or the complement thereof, corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; and

c) determining whether the extension product of the primer comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof.

25. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 mRNA molecule, or the complement thereof, that is proximate to a position corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID NO:12, or the complement thereof;

- 80 -

b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 mRNA molecule, or the complement thereof, corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID NO:12, or the complement thereof; and

c) determining whether the extension product of the primer comprises a uracil at a position corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID NO:12, or the complement thereof.

26. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 cDNA molecule, or the complement thereof, that is proximate to a position corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof; position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or position 3544 according to SEQ ID NO:22, or the complement thereof;

b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 cDNA molecule, or the complement thereof, corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof; position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or position 3544 according to SEQ ID NO:22, or the complement thereof; and

c) determining whether the extension product of the primer comprises a thymine at a position corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof; position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or position 3544 according to SEQ ID NO:22, or the complement thereof.

- 81 -

27. The method according to any one of claims 23 to 26, wherein the detecting step comprises sequencing the entire nucleic acid molecule.

28. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

5 a) amplifying at least a portion of the NOTCH3 genomic nucleic acid molecule, or the complement thereof, in the biological sample, wherein the portion comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof;

b) labeling the amplified nucleic acid molecule with a detectable label;

10 c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; and

15 d) detecting the detectable label.

29. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

a) amplifying at least a portion of the NOTCH3 mRNA molecule, or the complement thereof, in the biological sample, wherein the portion comprises a uracil at a position
20 corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID NO:12, or the complement thereof;

b) labeling the amplified nucleic acid molecule with a detectable label;

25 c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a uracil at a position corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID
30 NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID NO:12, or the complement thereof; and

- 82 -

d) detecting the detectable label.

30. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

a) amplifying at least a portion of the NOTCH3 cDNA molecule, or the complement thereof, produced from an mRNA molecule in the biological sample, wherein the portion comprises a thymine at a position corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof; position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or position 3544 according to SEQ ID NO:22, or the complement thereof;

b) labeling the amplified nucleic acid molecule with a detectable label;

c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof; position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or position 3544 according to SEQ ID NO:22, or the complement thereof; and

d) detecting the detectable label.

31. The method according to claim 31, wherein the nucleic acid molecule in the sample is mRNA and the mRNA is reverse-transcribed into cDNA prior to the amplifying step.

32. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

contacting the NOTCH3 genomic nucleic acid molecule, or the complement thereof, in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule, or the complement thereof, comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; and

detecting the detectable label.

- 83 -

33. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

contacting the NOTCH3 mRNA molecule, or the complement thereof, in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the NOTCH3 mRNA molecule, or the complement thereof, comprising a uracil at a position corresponding to: position 3781 according to SEQ ID NO:8, or the complement thereof; position 3767 according to SEQ ID NO:9, or the complement thereof; position 3532 according to SEQ ID NO:10, or the complement thereof; position 3769 according to SEQ ID NO:11, or the complement thereof; or position 3544 according to SEQ ID NO:12, or the complement thereof; and
detecting the detectable label.

34. The method according to any one of claims 16 to 20, wherein the detecting step comprises:

contacting the NOTCH3 cDNA molecule, or the complement thereof, produced from an mRNA molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the NOTCH3 cDNA molecule, or the complement thereof, comprising a thymine at a position corresponding to: position 3781 according to SEQ ID NO:18, or the complement thereof; position 3767 according to SEQ ID NO:19, or the complement thereof; position 3532 according to SEQ ID NO:20, or the complement thereof; position 3769 according to SEQ ID NO:21, or the complement thereof; or position 3544 according to SEQ ID NO:22, or the complement thereof; and
detecting the detectable label.

35. A method of treating a subject with a therapeutic agent that treats or inhibits a cerebrovascular disease, wherein the subject has a cerebrovascular disease, the method comprising:

determining whether the subject has a Neurogenic Locus Notch Homolog Protein 3 (NOTCH3) missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide by:
obtaining or having obtained a biological sample from the subject;
and

- 84 -

performing or having performed a sequence analysis on the biological sample to determine if the subject has a genotype comprising the NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide; and

5 administering or continuing to administer the therapeutic agent that treats or inhibits a cerebrovascular disease in a standard dosage amount to a subject that is NOTCH3 reference, and administering a NOTCH3 agent to the subject; and

administering or continuing to administer the therapeutic agent that treats or inhibits a cerebrovascular disease in an amount that is the same as or greater than a standard dosage
10 amount to a subject that is heterozygous or homozygous for the NOTCH3 missense variant nucleic acid molecule, and administering a NOTCH3 agent to the subject;

wherein the presence of a genotype having the NOTCH3 missense variant nucleic acid molecule encoding the NOTCH3 predicted gain-of-function polypeptide indicates the subject has an increased risk of developing a cerebrovascular disease.

15 36. The method according to claim 35, wherein the subject is NOTCH3 reference, and the subject is administered or continued to be administered the therapeutic agent that treats or inhibits the cerebrovascular disease in a standard dosage amount, and is administered a NOTCH3 agent.

37. The method according to claim 35, wherein the subject is heterozygous or
20 homozygous for a NOTCH3 missense variant nucleic acid molecule, and the subject is administered or continued to be administered the therapeutic agent that treats or inhibits the cerebrovascular disease in an amount that is the same as or greater than the standard dosage amount, and is administered a NOTCH3 agent.

38. The method according to any one of claims 35 to 37, wherein the NOTCH3 missense
25 variant nucleic acid molecule is a nucleic acid molecule encoding Arg1178Cys, Arg1231Cys, or Arg1182Cys.

39. The method according to any one of claims 35 to 37, wherein the NOTCH3 missense variant nucleic acid molecule is a nucleic acid molecule encoding Arg1231Cys.

40. The method according to claim 38, wherein the NOTCH3 missense variant nucleic acid
30 molecule is:

a genomic nucleic acid molecule having a nucleotide sequence comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2;

- 85 -

an mRNA molecule having a nucleotide sequence comprising a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12; or

5 a cDNA molecule produced from an mRNA molecule, wherein the cDNA molecule has a nucleotide sequence comprising a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22.

10 41. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof;

15 wherein when the sequenced portion of the NOTCH3 genomic nucleic acid molecule in the biological sample comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, then the NOTCH3 genomic nucleic acid molecule in the biological sample is a NOTCH3 missense variant genomic nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

20 42. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof;

25 wherein when the sequenced portion of the NOTCH3 mRNA molecule in the biological sample comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12, then the NOTCH3 mRNA molecule in the biological sample is a NOTCH3 missense variant mRNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

- 86 -

43. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 cDNA molecule produced from an mRNA molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to: position 3,781 according to SEQ ID NO:18, or
5 the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof;

wherein when the sequenced portion of the NOTCH3 cDNA molecule in the biological
10 sample comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22, then the NOTCH3 cDNA molecule in the biological sample is a NOTCH3 missense variant cDNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

15 44. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises:

a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule that is proximate to a position corresponding to position 21,944 according to SEQ ID NO:2;

20 b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule corresponding to position 21,944 according to SEQ ID NO:2; and

c) determining whether the extension product of the primer comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2.

25 45. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises:

a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 mRNA molecule that is proximate to a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID
30 NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12;

- 87 -

b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 mRNA molecule corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12; and

5 c) determining whether the extension product of the primer comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12.

46. The method according to any one of claims 35 to 40, wherein the sequence analysis
10 comprises:

a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 cDNA molecule that is proximate to a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or
15 position 3,544 according to SEQ ID NO:22;

b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 cDNA molecule corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22; and

20 c) determining whether the extension product of the primer comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22.

47. The method according to any one of claims 41 to 46, wherein the sequence analysis
25 comprises sequencing the entire nucleic acid molecule.

48. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises:

a) amplifying at least a portion of the genomic nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the portion comprises a thymine at a position corresponding to
30 position 21,944 according to SEQ ID NO:2, or the complement thereof;

b) labeling the amplified nucleic acid molecule with a detectable label;

- 88 -

c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to position
5 21,944 according to SEQ ID NO:2, or the complement thereof; and

d) detecting the detectable label.

49. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises:

a) amplifying at least a portion of the mRNA molecule that encodes the NOTCH3 polypeptide, wherein the portion comprises a uracil at a position corresponding to: position
10 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof;

15 b) labeling the amplified nucleic acid molecule with a detectable label;

c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a uracil at a position corresponding to: position
20 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; and

d) detecting the detectable label.

25 50. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises:

a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the portion comprises a thymine at a position corresponding to: position
30 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof;

- 89 -

- b) labeling the amplified nucleic acid molecule with a detectable label;
- c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof; and
- d) detecting the detectable label.
51. The method according to claim 50, wherein the nucleic acid molecule in the sample is mRNA and the mRNA is reverse-transcribed into cDNA prior to the amplifying step.
52. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises:
- contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; and
- detecting the detectable label.
53. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises:
- contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; and
- detecting the detectable label.

- 90 -

54. The method according to any one of claims 35 to 40, wherein the sequence analysis comprises:

contacting the nucleic acid molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a

5 nucleotide sequence which hybridizes under stringent conditions to
the nucleotide sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID
10 NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof; and

detecting the detectable label.

55. The method according to any one of claims 35 to 54, wherein the nucleic acid molecule is present within a cell obtained from the subject.

15 56. A method of identifying a subject having an increased risk for developing a cerebrovascular disease, the method comprising:

determining or having determined the presence or absence of a Neurogenic Locus Notch Homolog Protein 3 (NOTCH3) missense variant nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide in a biological sample obtained from the subject;

20 wherein:

when the subject is NOTCH3 reference, then the subject does not have an increased risk for developing the cerebrovascular disease; and

when the subject is heterozygous or homozygous for the NOTCH3 missense variant nucleic acid molecule, then the subject has an increased risk
25 for developing the cerebrovascular disease.

57. The method according to claim 56, wherein the NOTCH3 missense variant nucleic acid molecule is a nucleic acid molecule encoding Arg1178Cys, Arg1231Cys, or Arg1182Cys.

58. The method according to claim 56, wherein the NOTCH3 missense variant nucleic acid molecule is a nucleic acid molecule encoding Arg1231Cys.

30 59. The method according to claim 57, wherein the NOTCH3 missense variant nucleic acid molecule is:

- 91 -

a genomic nucleic acid molecule having a nucleotide sequence comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2;

an mRNA molecule having a nucleotide sequence comprising a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12; or

a cDNA molecule produced from an mRNA molecule, wherein the cDNA molecule has a nucleotide sequence comprising a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22.

60. The method according to any one of claims 56 to 59, wherein the determining step is carried out *in vitro*.

61. The method according to any one of claims 56 to 60, wherein the determining step comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof;

wherein when the sequenced portion of the NOTCH3 genomic nucleic acid molecule in the biological sample comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, then the NOTCH3 genomic nucleic acid molecule in the biological sample is a NOTCH3 missense variant genomic nucleic acid molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

62. The method according to any one of claims 56 to 60, wherein the determining step comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 mRNA molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof;

wherein when the sequenced portion of the NOTCH3 mRNA molecule in the biological sample comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID

- 92 -

NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12, then the NOTCH3 mRNA molecule in the biological sample is a NOTCH3 missense variant mRNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

- 5 63. The method according to any one of claims 56 to 60, wherein the determining step comprises sequencing at least a portion of the nucleotide sequence of the NOTCH3 cDNA molecule produced from an mRNA molecule in the biological sample, wherein the sequenced portion comprises a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof;

- wherein when the sequenced portion of the NOTCH3 cDNA molecule in the biological sample comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22, then the NOTCH3 cDNA molecule in the biological sample is a NOTCH3 missense variant cDNA molecule encoding a NOTCH3 predicted gain-of-function polypeptide.

64. The method according to any one of claims 56 to 60, wherein the determining step comprises:

- a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule that is proximate to a position corresponding to position 21,944 according to SEQ ID NO:2;
- b) extending the primer at least through the position of the nucleotide sequence of the NOTCH3 genomic nucleic acid molecule corresponding to position 21,944 according to SEQ ID NO:2; and

c) determining whether the extension product of the primer comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2.

65. The method according to any one of claims 56 to 60, wherein the determining step comprises:

a) contacting the biological sample with a primer hybridizing to a portion of the nucleotide sequence of the NOTCH3 mRNA molecule that is proximate to a position

- 93 -

corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10; position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12;

b) extending the primer at least through the position of the nucleotide sequence of the
5 NOTCH3 mRNA molecule corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12; and

c) determining whether the extension product of the primer comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, position 3,767 according to
10 SEQ ID NO:9, position 3,532 according to SEQ ID NO:10, position 3,769 according to SEQ ID NO:11, or position 3,544 according to SEQ ID NO:12.

66. The method according to any one of claims 56 to 60, wherein the determining step comprises:

a) contacting the biological sample with a primer hybridizing to a portion of the
15 nucleotide sequence of the NOTCH3 cDNA molecule that is proximate to a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, position 3,544 according to SEQ ID NO:22;

b) extending the primer at least through the position of the nucleotide sequence of the
20 NOTCH3 cDNA molecule corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22; and

c) determining whether the extension product of the primer comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, position 3,767 according
25 to SEQ ID NO:19, position 3,532 according to SEQ ID NO:20, position 3,769 according to SEQ ID NO:21, or position 3,544 according to SEQ ID NO:22.

67. The method according to any one of claims 61 to 66, wherein the determining step comprises sequencing the entire nucleic acid molecule.

68. The method according to any one of claims 56 to 60, wherein the determining step
30 comprises:

- 94 -

a) amplifying at least a portion of the nucleic acid molecule that encodes the NOTCH3 polypeptide, wherein the portion comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof;

b) labeling the amplified nucleic acid molecule with a detectable label;

5 c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; and

10 d) detecting the detectable label.

69. The method according to any one of claims 56 to 60, wherein the determining step comprises:

a) amplifying at least a portion of the mRNA molecule that encodes the NOTCH3 polypeptide, wherein the portion comprises a uracil at a position corresponding to: position 15 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof;

b) labeling the amplified nucleic acid molecule with a detectable label;

20 c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID 25 NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; and

d) detecting the detectable label.

70. The method according to any one of claims 56 to 60, wherein the determining step 30 comprises:

a) amplifying at least a portion of the cDNA molecule that encodes the NOTCH3 polypeptide, wherein the portion comprises a thymine at a position corresponding to: position

- 95 -

3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof

- 5 b) labeling the amplified nucleic acid molecule with a detectable label;
- c) contacting the labeled nucleic acid molecule with a support comprising an alteration-specific probe, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleic acid sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to: position
- 10 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof; and
- d) detecting the detectable label.

- 15 71. The method according to claim 70, wherein the nucleic acid molecule in the sample is mRNA and the mRNA is reverse-transcribed into cDNA prior to the amplifying step.

72. The method according to any one of claims 56 to 60, wherein the detecting step comprises:

- contacting the genomic nucleic acid molecule in the biological sample with an
- 20 alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof; and
- detecting the detectable label.

- 25 73. The method according to any one of claims 56 to 60, wherein the detecting step comprises:

- contacting the mRNA molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of
- 30 the amplified nucleic acid molecule comprising a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the

- 96 -

complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; and detecting the detectable label.

74. The method according to any one of claims 56 to 60, wherein the detecting step
5 comprises:
contacting the cDNA molecule produced from the mRNA molecule in the biological sample with an alteration-specific probe comprising a detectable label, wherein the alteration-specific probe comprises a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of the amplified nucleic acid molecule comprising a thymine at a
10 position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof; and
15 detecting the detectable label.

75. The method according to any one of claims 56 to 74, wherein the subject is NOTCH3 reference, and the subject is administered a therapeutic agent that treats or inhibits a cerebrovascular disease in a standard dosage amount, and is administered a NOTCH3 agent.

76. The method according to any one of claims 56 to 74, wherein the subject is
20 heterozygous or homozygous for the NOTCH3 missense variant nucleic acid molecule, and the subject is administered a therapeutic agent that treats or inhibits a cerebrovascular disease in an amount that is the same as or greater than a standard dosage amount, and is administered a NOTCH3 agent.

77. A therapeutic agent that treats or inhibits a cerebrovascular disease for use in the
25 treatment of a cerebrovascular disease in a subject identified as having:
a genomic nucleic acid molecule having a nucleotide sequence encoding a Neurogenic Locus Notch Homolog Protein 3 (NOTCH3) polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof;

- 30 an mRNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID

- 97 -

NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; or

a cDNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide,

5 wherein the nucleotide sequence comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof.

10 78. A Neurogenic Locus Notch Homolog Protein 3 (NOTCH3) agent for use in the treatment of a cerebrovascular disease in a subject identified as having:

a genomic nucleic acid molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to position 21,944 according to SEQ ID NO:2, or the complement thereof;

15 an mRNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a uracil at a position corresponding to: position 3,781 according to SEQ ID NO:8, or the complement thereof; position 3,767 according to SEQ ID NO:9, or the complement thereof; position 3,532 according to SEQ ID NO:10, or the complement thereof; position 3,769 according to SEQ ID NO:11, or the complement thereof; or position 3,544 according to SEQ ID NO:12, or the complement thereof; or

20 a cDNA molecule having a nucleotide sequence encoding a NOTCH3 polypeptide, wherein the nucleotide sequence comprises a thymine at a position corresponding to: position 3,781 according to SEQ ID NO:18, or the complement thereof; position 3,767 according to SEQ ID NO:19, or the complement thereof; position 3,532 according to SEQ ID NO:20, or the complement thereof; position 3,769 according to SEQ ID NO:21, or the complement thereof; or position 3,544 according to SEQ ID NO:22, or the complement thereof.

79. The NOTCH3 agent according to claim 78, which is an inhibitory nucleic acid molecule.

80. The NOTCH3 agent according to claim 79, wherein the inhibitory nucleic acid molecule is an antisense nucleic acid molecule, a small interfering RNA (siRNA), or a short hairpin RNA (shRNA) that hybridizes to a NOTCH3 nucleic acid molecule.

30

- 98 -

81. The NOTCH3 agent according to claim 78, which comprises a Cas protein and guide RNA (gRNA) that hybridizes to a gRNA recognition sequence within a NOTCH3 genomic nucleic acid molecule.
82. The NOTCH3 agent according to claim 81, wherein the Cas protein is Cas9 or Cpf1.
- 5 83. The NOTCH3 agent according to claim 81 or claim 82, wherein the gRNA recognition sequence includes or is proximate to position 21,944 according to SEQ ID NO:2.
84. The NOTCH3 agent according to claim 81 or claim 82, wherein the gRNA recognition sequence is located about 1000, about 500, about 400, about 300, about 200, about 100, about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 15, about 10, or about 5
- 10 nucleotides from a position corresponding to position 21,944 according to SEQ ID NO:2.
85. The NOTCH3 agent according to claim 81 or claim 82, wherein a Protospacer Adjacent Motif (PAM) sequence is located about 2 to about 6 nucleotides downstream of the gRNA recognition sequence.
86. The NOTCH3 agent according to any one of claims 81 to 85, wherein the gRNA
- 15 comprises from about 17 to about 23 nucleotides.
87. The NOTCH3 agent according to any one of claims 81 to 86, wherein the gRNA recognition sequence comprises a nucleotide sequence according to any one of SEQ ID NOs:29-43.