# Antibodies against human EPO Receptor

## Background of the Invention

Human erythropoietin (EPO) is a 166-aa glycoprotein which is involved in the proliferation and differentiation of erythroid progenitor cells. These cellular responses are mediated by the human EPO receptor (EPO-R), a 508-aa glycoprotein. Human EPO receptor (EPO-R) is a protein of 508 amino acid length (Swiss Prot P19235) containing a single transmembrane domain and has been classified as a member of the growth hormone subfamily of class I cytokine receptors. EPO-R is e. g. described in Winkelmann, J.C. et al., Blood 76 (1990) 24-30 and Jones, S.S. et al., Blood 76 (1990) 31-35. Activation of EPO-R occurs by dimerization (Matthews, D.J., PNAS 93 (1996) 9471-9476). EPO-R comprises eight cytoplasmic tyrosine sites which become phosphorylated upon stimulation with EPO (Li, K. et al., J. Biol. Chem. 278 (2003) 40702-40709; Wu, H.et al., Proc. Natl. Acad. Sci. USA 94 (1997) 1806-1810) resulting in "activated EPO-R".

Antibodies against EPO-R are e.g. known from Andrea, A.D., Blood 82 (1993) 46-52; Elliott, S., Blood 107 (2006) 1892-1895; Kirkeby, A., J. Nerosci. 164 (2007) 50-58; Miura, O., Arch. Biochem. 306 (1993) 200-208; and EP 1 146 056, EP 1 327 681, EP 0 773 962, EP 0 776 370, US 2002/0031806, US 2003/0215444, US 2004/0058393, US 2004/0071694, US 2004/0175379, US 2005/0227289, US 2005/0244409, US 2006/0018902, US 6,998,124, US 7,053,184, US 7,081,523, WO 1995/005469, WO 1996/003438, WO 2000/061637, WO 2004/035603 A2, WO 2005/100403 A2. However it is known form the state of the art, that known antibodies against EPO-R are not able to discriminate between non activated and activated EPO-R (see Jelkmann, W. et al., Crit. Rev. Onc/Hematol. 67 (2008) 39-61; Li, K. et al., J. Biol. Chem. 278 (2003) 40702-40709, and Wu, H. et al., Proc. Natl. Acad. Sci. USA 94 (1997) 1806-1810).

## Summary

The disclosure comprises an antibody specifically binding to activated human EPO receptor and discriminating between non activated and activated EPO-R, which allows specific analysis of the activation of EPO-R especially in cells and biopsies from human tissue.

The disclosure comprises an antibody, characterized in specifically binding human EPO receptor fragment TPPHLKYLYLVVSD (SEQ ID NO:25) comprising a phospho-tyrosyl residue at position 430 (underlined), GLSDGPYSNPYENSLIP (SEQ ID NO:26) comprising a phospho-tyrosyl residue at position 461 (underlined), or GLSDGPYSNPYENSLIP (SEQ ID NO:26) comprising a phospho-tyrosyl residue at position 465 (underlined).

Numbering relates to EPO receptor amino acid sequence of UniProtKB/Swiss-Prot P19235 without signal peptide.

In one aspect, there is provided an antibody binding to human EPO receptor, characterized in specifically binding EPO receptor fragment GLSDGPYSNPYENSLIP (SEQ ID NO:26) comprising a phospho-tyrosyl residue at position 465, whereby the antibody specifically binds to activated human EPO receptor and discriminates between non activated and activated EPO-R, the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:17, a CDR2 region of SEQ ID NO:18 and a CDR1 region of SEQ ID NO:19 and the light chain variable domain comprises a CDR3 region of SEQ ID NO:20, a CDR2 region of SEQ ID NO:21 and a CDR1 region of SEQ ID NO: 22, and whereby the antibody is specifically binding pY465 in ELISA at a S/N ratio of 10 or more at an antibody concentration of 0.1µg/ml.

An antibody according to the disclosure does not bind EPO receptor fragment TPPHLKYLYLVVSD (SEQ ID NO:25) without a phospho-tyrosyl residue at position 430, GLSDGPYSNPYENSLIP (SEQ ID NO:26) without a phospho-tyrosyl residue at position 461, or GLSDGPYSNPYENSLIP (SEQ ID NO:26) without a phospho-tyrosyl residue at position 465.

An antibody according to the disclosure specifically binds phosphorylated (activated) EPO receptor in lysates of UT7 cells which cells are expressing EPO-R in an amount of 100.000 to 500.000 receptors per cell (EPO-R expressing cells) and being treated with 500 pM EPO for activation. An antibody according to the invention does not bind EPO receptor in lysates of UT7 cells expressing EPO receptor and being not treated with EPO. Such binding can be measured by Western Blot.

Preferably the disclosure comprises an antibody binding to human EPO-R, characterized in comprising as heavy chain variable domain CDR3 region a CDR3 region of SEQ ID NO: 1, 9 or 17.

Preferably the antibody is characterized in that the heavy chain variable domain comprises CDR3 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO:2 and a CDR1 region of SEQ ID NO:3 or CDR3 region of SEQ ID NO:9, a CDR2 region of SEQ ID NO:10 and a CDR1 region of SEQ ID NO:11 or CDR3 region of SEQ ID NO:17, a CDR2 region of SEQ ID NO:18 and a CDR1 region of SEQ ID NO:19.

Preferably the antibody is characterized in that the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO:2 and a CDR1 region of SEQ ID NO:3 and in that the light chain variable domain

comprises a CDR3 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5 and a CDR1 region of SEQ ID NO:6.

Preferably the antibody is characterized in that the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 9, a CDR2 region of SEQ ID NO:10 and a CDR1 region of SEQ ID NO:11 and in that the light chain variable domain comprises a CDR3 region of SEQ ID NO: 12, a CDR2 region of SEQ ID NO:13 and a CDR1 region of SEQ ID NO:14.

Preferably the antibody is characterized in that the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO:18 and a CDR1 region of SEQ ID NO:19 and in that the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO:21 and a CDR1 region of SEQ ID NO:22.

Preferably the antibody is characterized in that the heavy chain variable domain comprises SEQ ID NO:7, 15 or 23.

Preferably the antibody is characterized in that the light chain variable domain comprises SEQ ID NO:8, 16 or 24.

Preferably the antibody is characterized in that the heavy chain variable domain comprises SEQ ID NO:7 and the light chain variable domain comprises SEQ ID NO:8.

Preferably the antibody is characterized in that the heavy chain variable domain comprises SEQ ID NO:15 and the light chain variable domain comprises SEQ ID NO:16.

Preferably the antibody is characterized in that the heavy chain variable domain comprises SEQ ID NO:23 and the light chain variable domain comprises SEQ ID NO:24.

Preferably the antibody according to the disclosure is characterized in binding to EPO-R with a binding affinity of at least $10^{-8}$ $M^{-1}$ to $10^{-12}$ $M^{-1}$ .

A further embodiment of the disclosure is a nucleic acid encoding a heavy chain and a light chain of an antibody according to the disclosure.

Human and other constant chains are well known in the state of the art and e.g. described by Kabat (see e.g. Johnson, G. and Wu, T.T., Nucleic Acids Res. 28 (2000) 214-218). It is further preferred that the antibody is of mouse origin and comprises the antibody variable sequence frame of a mouse antibody according to Kabat (see e.g. Johnson, G. and Wu, T.T., Nucleic Acids Res. 28 (2000) 214-218).

The antibody according to the disclosure is preferably of mouse, rabbit or human origin. As human antibody isotype IgG1 is preferred.

The disclosure further provides expression vectors containing nucleic acid according to the disclosure capable of expressing said nucleic acid in a prokaryotic or eukaryotic host cell, and host cells containing such vectors for the recombinant production of such an antibody.

The disclosure further comprises a prokaryotic or eukaryotic host cell comprising a vector according to the disclosure.

The disclosure further comprises a method for the production of a recombinant human or humanized antibody according to the disclosure, characterized by expressing a nucleic acid according to the disclosure in a prokaryotic or eukaryotic host cell and recovering said antibody from said cell or the cell culture supernatant. The disclosure further comprises the antibody obtainable by such a recombinant method.

The disclosure further comprises the use of an antibody according to the disclosure to determine/detect mammalian cells bearing/expressing activated EPO receptor.

In another aspect, there is provided the use of an antibody as defined above for the *in vitro* determination of activated EPO receptor in human cells tissues, and biopsies.

Preferably an antibody according to the disclosure is used to determine activated EPO receptor in lysates of biopsies of human tissue samples. Preferably such detection is performed by Western Blot.

**Detailed Description of the Invention**

The term "antibody" encompasses the various forms of antibody structures including but not being limited to whole antibodies and antibody fragments.

"Antibody fragments" comprise a portion of a full length antibody, preferably the variable domain thereof, or at least the antigen binding site thereof. Examples of antibody fragments include diabodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. scFv antibodies are, e.g.,

described in Houston, J.S., Methods Enzymol. 203 (1991) 46-88. In addition, antibody fragments comprise single chain polypeptides having the characteristics of a $V_H$ domain, namely being able to assemble together with a $V_L$ domain, or of a $V_L$ domain binding to EPO-R, namely being able to assemble together with a $V_H$ domain to a functional antigen binding site and thereby providing an antibody with the properties of specifically binding to human EPO-R.

The term "humanized antibody" refers to antibodies in which the framework and/or "complementarity determining regions" (CDR) have been modified to comprise the CDR of an immunoglobulin of different species as compared to that of the parent immunoglobulin. In a preferred embodiment, a mouse CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody". See, e.g., Riechmann, L. et al., Nature 332 (1988) 323-327; and Neuberger, M.S. et al., Nature 314 (1985) 268-270.

The term "comprises a heavy chain CDR3 region of SEQ ID NO:1" denotes that the antibody comprises as sequence of its heavy chain CDR3 region the amino acid sequence of SEQ ID NO:1. The same denotes for the other five CDR regions of the antibody.

The term "binding to activated EPO-R" as used herein means binding of the antibody to human activated EPO-R in a biochemical binding assay measured by Western Blotting. Binding is found if the antibody causes an S/N (signal/noise) ratio of 400 or more at an antibody concentration of 1µg/ml. The term "not binding to EPO-R" as used herein means binding of the antibody to human EPO-R in a biochemical binding assay measured by Western Blotting. No binding is found if the antibody causes an S/N (signal/noise) ratio of lower than 400 at an antibody concentration of 1µg/ml. Binding of the antibodies according to the invention to non activated EPO R is not detectable in Western Blots, therefore the S/N ratio is even lower than 10 and preferably about 1 or lower.

The term "binding of EPO to EPO receptor" as used herein means binding of the EPO to human activated EPO-R in a biochemical binding assay measured by Western Blotting. No binding is found if EPO causes an S/N (signal/noise) ratio of no more than 10 at an EPO concentration of 1µg/ml.

The term "pY430" as used herein means a 17 amino-acid synthetic peptide corresponding to residues 424-437 of the mature human erythropoietin receptor (TPPHLKYLYLVVSD, SEQ ID NO:25), comprising a phospho-tyrosyl residue at

position 430.The term "pY461" as used herein means a 17 amino-acid synthetic peptide corresponding to residues 455-471 of the mature human erythropoietin receptor (GLSDGPYSNPYENSLIP, SEQ ID NO:26), comprising a phospho-tyrosyl residue at position 461. The term "pY465" as used herein means a 17 amino-acid synthetic peptide corresponding to residues 455-471 of the mature human erythropoietin receptor (GLSDGPYSNPYENSLIP, SEQ ID NO:26), comprising a phospho-tyrosyl residue at position 465.

The antibody according to the invention is characterized in specifically binding pY430, pY461 or pY465 in ELISA at a S/N ratio of 10 or more at an antibody concentration of $0.1\mu g/ml$ .

The "variable domain" (variable domain of a light chain ($V_L$), variable domain of a heavy chain ($V_H$)) as used herein denotes each of the pair of light and heavy chain domains which are involved directly in binding the antibody to the antigen. The variable light and heavy chain domains have the same general structure and each domain comprises four framework (FR) regions whose sequences are widely conserved, connected by three "hypervariable regions" (or complementarity determining regions, CDRs). The framework regions adopt a β-sheet conformation and the CDRs may form loops connecting the β-sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the antigen binding site. The antibody's heavy and light chain CDR3 regions play a particularly important role in the binding specificity/affinity of the antibodies according to the invention and therefore provide a further object of the invention.

The term "antigen-binding portion of an antibody" when used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The antigen-binding portion of an antibody comprises amino acid residues from the "complementarity determining regions" or "CDRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chain variable domains of an antibody comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding and defines the antibody's properties. CDR and FR regions are determined according to the standard definition of Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service,

National Institutes of Health, Bethesda, MD (1991) and/or those residues from a "hypervariable loop".

The terms "nucleic acid" or "nucleic acid molecule", as used herein, are intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "amino acid" as used within this application denotes the group of naturally occurring carboxy α-amino acids comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are colinear, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived there from without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

Examples of useful human heavy chain constant region comprises an amino acid of SEQ ID NO: 23. For example an useful human light chain constant region

comprises an amino acid sequence of a kappa-light chain constant region of SEQ ID NO: 24.

The invention comprises a method for detecting or determination of activated EPO-R in human cells tissues, and biopsies.

The invention comprises the use of an antibody according to the invention for diagnosis of activated EPO-R in human cells tissues, and biopsies.

The invention comprises the use of an antibody according to the invention for the preparation of a diagnostis assay for detecting or determining activated EPO-R in human cells tissues, and biopsies.

The antibodies according to the invention include, in addition, such antibodies having "conservative sequence modifications" (variant antibodies), nucleotide and amino acid sequence modifications which do not affect or alter the above-mentioned characteristics of the antibody according to the invention. Modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g. lysine, arginine, histidine), acidic side chains (e.g. aspartic acid, glutamic acid), uncharged polar side chains (e.g. glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g. alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g. threonine, valine, isoleucine) and aromatic side chains (e.g. tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a human anti- EPO-R antibody can be preferably replaced with another amino acid residue from the same side chain family. A "variant" anti-EPO-R antibody, refers therefore herein to a molecule which differs in amino acid sequence from a "parent" anti- EPO-R antibody amino acid sequence by up to ten, preferably from about two to about five, additions, deletions and/or substitutions in one or more variable region of the parent antibody. Amino acid substitutions can be performed by mutagenesis based upon molecular modeling as described by Riechmann, L. et al., Nature 332 (1988) 323-327 and Queen, C. et al., Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033.

The antibodies according to the invention are preferably produced by recombinant means. Such methods are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody polypeptide and usually purification to a pharmaceutically acceptable purity. For the protein expression nucleic acids encoding light and heavy chains or fragments thereof are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells, such as CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, yeast, or E. coli cells, and the antibody is recovered from the cells (from the supernatant or after cells lysis).

Recombinant production of antibodies is well-known in the state of the art and described, for example, in the review articles of Makrides, S.C., Protein Expr. Purif. 17 (1999) 183-202; Geisse, S. et al., Protein Expr. Purif. 8 (1996) 271-282; Kaufman, R.J., Mol. Biotechnol. 16 (2000) 151-161; Werner, R.G., Drug Res. 48 (1998) 870-880.

The antibodies may be present in whole cells, in a cell lysate, or in a partially purified, or substantially pure form. Purification is performed in order to eliminate other cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See Ausubel, F., et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).

Expression in NS0 cells is described by, e.g., Barnes, L.M., et al., Cytotechnology 32 (2000) 109-123; Barnes, L.M., et al., Biotech. Bioeng. 73 (2001) 261-270. Transient expression is described by, e.g., Durocher, Y., et al., Nucl. Acids. Res. 30 (2002) E9. Cloning of variable domains is described by Orlandi, R., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 3833-3837; Carter, P., et al., Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; Norderhaug, L., et al., J. Immunol. Methods 204 (1997) 77-87. A preferred transient expression system (HEK 293) is described by Schlaeger, E.-J. and Christensen, K., in Cytotechnology 30 (1999) 71-83, and by Schlaeger, E.-J., in J. Immunol. Methods 194 (1996) 191-199.

Monoclonal antibodies are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or

affinity chromatography. DNA and RNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures. The hybridoma cells can serve as a source of such DNA and RNA. Once isolated, the DNA may be inserted into expression vectors, which are then transfected into host cells, such as HEK 293 cells, CHO cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of recombinant monoclonal antibodies in the host cells.

Amino acid sequence variants of human EPO-R antibody are prepared by introducing appropriate nucleotide changes into the antibody encoding DNA, or by peptide synthesis. Such modifications can be performed, however, only in a very limited range, e.g. as described above. For example, the modifications do not alter the abovementioned antibody characteristics such as the IgG isotype and epitope binding, but may improve the yield of the recombinant production, protein stability, or facilitate the purification.

Any cysteine residue not involved in maintaining the proper conformation of the anti- EPO-R antibody may also be substituted, generally with serine, to improve the oxidative stability of the molecule and to prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

Nucleic acid molecules encoding amino acid sequence variants of anti- EPO-R antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of humanized anti- EPO-R antibody.

The heavy and light chain variable domains according to the invention are combined with sequences of promoter, translation initiation, constant region, 3' untranslated region, polyadenylation, and transcription termination to form expression vector constructs. The heavy and light chain expression constructs can be combined into a single vector, co-transfected, serially transfected, or separately transfected into host cells which are then fused to form a single host cell expressing both chains.

In another aspect, the present invention provides a diagnostic composition, e.g. for the determination of activated EPO-R in human cells tissues, and biopsies.

**Description of the Sequences**

| | |
|---|---|
| SEQ ID NO: 1 | heavy chain CDR3, Mab Cl.16.7.5 |
| SEQ ID NO: 2 | heavy chain CDR2, Mab Cl.16.7.5 |
| SEQ ID NO: 3 | heavy chain CDR1, Mab Cl.16.7.5 |
| SEQ ID NO: 4 | light chain CDR3, Mab Cl.16.7.5 |
| SEQ ID NO: 5 | light chain CDR2, Mab Cl.16.7.5 |
| SEQ ID NO: 6 | light chain CDR1, Mab Cl.16.7.5 |
| SEQ ID NO: 7 | heavy chain variable domain, Mab Cl.16.7.5 |
| SEQ ID NO: 8 | light chain variable domain , Mab Cl.16.7.5 |
| SEQ ID NO: 9 | heavy chain CDR3, Mab Cl.8.7.16 |
| SEQ ID NO: 10 | heavy chain CDR2, Mab Cl.8.7.16 |
| SEQ ID NO: 11 | heavy chain CDR1, Mab Cl.8.7.16 |
| SEQ ID NO: 12 | light chain CDR3, Mab Cl.8.7.16 |
| SEQ ID NO: 13 | light chain CDR2, Mab Cl.8.7.16 |
| SEQ ID NO: 14 | light chain CDR1, Mab Cl.8.7.16 |
| SEQ ID NO: 15 | heavy chain variable domain, Mab Cl.8.7.16 |
| SEQ ID NO: 16 | light chain variable domain , Mab Cl.8.7.16 |
| SEQ ID NO: 17 | heavy chain CDR3, Mab Cl.24.11.31 |
| SEQ ID NO: 18 | heavy chain CDR2, Mab Cl.24.11.31 |
| SEQ ID NO: 19 | heavy chain CDR1, Mab Cl.24.11.31 |
| SEQ ID NO: 20 | light chain CDR3, Mab Cl.24.11.31 |
| SEQ ID NO: 21 | light chain CDR2, Mab Cl.24.11.31 |
| SEQ ID NO: 22 | light chain CDR1, Mab Cl.24.11.31 |
| SEQ ID NO: 23 | heavy chain variable domain, Mab Cl.24.11.31 |
| SEQ ID NO: 24 | light chain variable domain , Mab Cl.24.11.31 |
| SEQ ID NO: 25 | synthetic peptide |
| SEQ ID NO: 26 | synthetic peptide |

**Description of the Figures**

**Figure 1:** Time dependent phosphorylation of human EPOR in UT-7_EPOR cells. Western blot analysis of lysates of UT7-EPOR cells stained with the different monoclonal antibodies directed

against phosphorylated human EPOR. (a) pY430 (Cl.16.7.5), (b) pY461 (Cl.8.7.16), (c) pY465 (Cl.24.11.31). All monoclonal antibodies specifically detect EPOR only when cells are stimulated with EPO, unstimulated cells (-) are not labeled by the antibodies. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as loading control.

**Example 1**

**Generation of phospho-specific EPOR monoclonal antibodies**

pY430 (Cl.16.7.5): a 17 amino-acid synthetic peptide corresponding to residues 424-437 of the mature human erythropoietin receptor (TPPHLKYLYLVVSD, SEQ ID NO:25), comprising a phospho-tyrosyl residue at position 430 was used as immunogen.

pY461 (Cl.8.7.16): a 17 amino-acid synthetic peptide corresponding to residues 455-471 of the mature human erythropoietin receptor (GLSDGPYSNPYENSLIP, SEQ ID NO:26), comprising a phospho-tyrosyl residue at position 461 was used as immunogen.

pY465 (Cl.24.11.31): a 17 amino-acid synthetic peptide corresponding to residues 455-471 of the mature human erythropoietin receptor (GLSDGPYSNPYENSLIP, SEQ ID NO:26), comprising a phospho-tyrosyl residue at position 465 was used as immunogen.

For immunization the peptides were coupled to KLH via a C terminal cystein. Balb/c mice were immunized with immunogen every four weeks for 3 times followed by an i.v. boost on day 4 before fusion, splenocytes were harvested, and fused with Ag8 myeloma cells. Screening for phospho-specific antibodies was done by differential testing on phospho –vs. non-phospho form of the peptide (which otherwise was identical to the phospho-peptide) using peptide coated ELISA microtiter plates following standard procedues. Antibody clones were selected because they detected one specific band corresponding to the EPOR on Western Blots of cell lysates that have been stimulated with EPO (example 2).

**SDS-PAGE and Western blotting:**

The SDS-PAGE and Western blotting were performed according to standard procedures and the Nupage® gel system of Invitrogen. The lysates corresponding to $5.10^4$-$10^5$ cells were loaded in each line of a Nupage® Novex 4-12% Bis-Tris

gel. The proteins were then transferred onto PVDF (Polyvinylidene Fluoride) membranes and incubated with the antibodies and anti-GAPDH antibody (Abcam m9484, Abcam plc UK)) for 2 hours at room temperature or overnight at 4°C . After washing, the membranes were incubated with a conjugate anti-mouse IgG-POD and developed using ECL reagents (Lumi-LightPLUS Western blotting substrate, Roche Applied Science #2015218).

Antibodies Cl.24.11.31, Cl.16.7.5 and Cl.8.7.16 were selected as binding specifically to activated EPO receptor only without binding to non-activated EPO receptor (Fig.1).

**Example 2**

**Activation of UT-7 cells**

UT-7 cell line is a human factor-dependent erythroleukemic cell line (Human bone marrow acute myeloid leukemia cell line DSMZ: ACC 137), requiring EPO for long-term growth. UT7 cells were maintained in RPMI medium comprising in addition L-glutamine (2mM), non-essential amino acids (1x), and sodium pyruvate (1mM) (starvation medium), supplemented with 10% fetal calf serum and 10U/ml GM-CSF. Transduced cells (UT7/EPOR) were maintained in the same medium as untransduced (25U/ml GM-CSF instead of 10U/ml) with the addition of 0.4 mg/ml zeocine. Before each stimulation the cells were starved by incubation overnight in RPMI media supplemented with L-glutamine (2mM), non-essential amino acids (1x), sodium pyruvate (1mM) and 0.1% fetal calf serum.

**Transduction:**

UT-7 cells were transduced with the supernatant from GP2-293 (Clontech Laboratories, Inc) cells transiently transfected with a retroviral expression vector encoding hEPOR and pVSV-G (an expression vector encoding the G glycoprotein of the rhabdovirus vesicular stomatitis virus). Two days after transduction the medium was replaced with fresh supplemented RPMI containing 0.4 mg/ml zeocine and 25U/ml GM-CSF. After selection a cell line of UT-7 cells stable expressing EPOR on their surface was obtained.

**Stimulation:**

Serum starved cells were stimulated for the times indicated with 500 pM erythropoietin in starvation medium (supplemented with 0.1% fetal calf serum) at 37°C for 1, 3 and 5 minutes. After stimulation the cells were centrifuged, the medium was discarded and the pellet was incubated in ice-cold lysis buffer [Tris

20 mM (pH7.4), NaCl 137 mM, Glycerol 10%, Nonidet® P-40 1% , protease inhibitors 1x (Pierce, # 78410), phosphatase inhibitors 1x (Pierce #78420)] for 30 minutes at 4°C followed by centrifugation at 13000 rpm for 10 minutes at 4°C. The lysate supernatant was boiled in sample buffer (Nupage®, Invitrogen) in the presence of a reducing agent and either directly used for SDS-PAGE.

Specific binding of the Mabs was tested by ELISA on microtiterplates coated with the corresponding biotinylated peptide according to standard procedures as described in example1. Already within 1 min a clear activation of human EPOR can be demonstrated while un-stimulated cells do not show any detectable basal level of activation.

**Example 3**

**Western Blot assay for binding of Mabs to UT-7/EPOR cells activated by EPO**

UT7/EPOR cells were stimulated as described above and analyzed by Western Blot. On Western blot the Mabs recognize one band at MW 66kD corresponding to the EPOR (Fig.1) in cells that are stimulated with 0.5nM EPO for 1-5 min. Already within 1 min a clear activation of human EPOR can be demonstrated while un-stimulated cells do not show any detectable basal level of activation (Fig.1). As loading control GAPDH was used. Thus, these antibodies specifically analyze the activation of EPOR by ESAs (erythropoietin stimulating agents).

**Patent Claims**

1. Antibody binding to human EPO receptor, characterized in specifically binding EPO receptor fragment GLSDGPYSNPYENSLIP (SEQ ID NO:26) comprising a phospho-tyrosyl residue at position 465,

whereby the antibody specifically binds to activated human EPO receptor and discriminates between non activated and activated EPO-R, the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:17, a CDR2 region of SEQ ID NO:18 and a CDR1 region of SEQ ID NO:19 and the light chain variable domain comprises a CDR3 region of SEQ ID NO:20, a CDR2 region of SEQ ID NO:21 and a CDR1 region of SEQ ID NO: 22, and

whereby the antibody is specifically binding pY465 in ELISA at a S/N ratio of 10 or more at an antibody concentration of 0.1µg/ml.

2. Antibody according to claim 1, characterized in that the heavy chain variable domain comprises SEQ ID NO:23 and the light chain variable domain comprises SEQ ID NO:24.

3. Use of an antibody according to claims 1 to 2 for the *in vitro* determination of activated EPO receptor in human cells tissues, and biopsies.

4. Use according to claim 3, characterized in that the human cells tissue is a lysate of human tissue.

5. Use according to claim 4, characterized in that the determination is performed by Western Blot or ELISA