

ANIMAL MODEL FOR AMPLIFYING HUMAN OR ANIMAL CIRCULATING TUMOUR CELLS

FIELD OF THE INVENTION

5 The present invention relates to an avian model enabling the amplification of human or animal circulating tumour cells (CTC).

PRIOR ART

10 CTC are cells that detach from a tumour and which are capable of being displaced to pass into the blood, after having joined the vascular system. These cells can thus migrate to other organs and participate in the development of metastases. These cells thus represent a very attractive target in the monitoring of the evolution and the treatment of cancers, or even in the discovery of novel therapeutics.

15 The isolation of CTC is not however an easy thing because these cells are very few in number. It is thus necessary to expand them before being able to study them. At present, CTC can be amplified using *in vitro* or *in vivo* cultures in murine models (Kowalik A *et al.*, 2017; Drapkin B. J. *et al.*, 2018). *In vitro* cultures of CTC derived from numerous cancers (lung cancer, prostate cancer, breast cancer, urinary tract cancer, head cancer or neck cancer, etc.) have already been produced, making it
20 possible to conduct high speed preclinical tests of therapeutic regimes, but with limited success because these cultures are too long to be compatible with clinical practice and expose the CTC to phenotype modifications (Pantel K, Alix-Panabières C., 2016).

25 Short term *in vivo* cultures have moreover been established in a murine model, from CTC, and after a first step of *in vitro* expansion when the amount of CTC was too low (Giuliano M *et al.*, 2015; Williams ES *et al.*, 2015; Torphy RJ *et al.*, 2014; Rossi E *et al.*, 2013). The direct inoculation of CTC in immunodeficient mice has also been envisaged as a method for amplifying isolated CTC. In highly metastatic small cell lung cancers (SCLC), CTC isolated in patients having a high level of CTC (> 400
30 CTC per 7.5 mL of blood) formed tumours in mice for which the response to chemotherapies with platinum and with Etoposide corresponded to the clinical

observations (Hodgkinson, C. L. *et al.*, 2014). These studies open the way to the use of immunodeficient mice as "incubators" of CTC, capable of generating preclinical models adapted to the individual tumours of patients.

However, these models are too expensive to envisage the systematic use thereof.

5 They frequently require having to carry out a prior step of *in vitro* expansion with the aforementioned drawbacks. Finally, the establishment of these models requires time, which is not always compatible with the observation of the desired effects.

There thus exists a need to find a less expensive *in vivo* model, but also which gives results more rapidly, and in particular which does not necessitate a prior *in vitro* expansion phase in order to work on these CTC which are the closest to those present *in vivo* in patients.

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SUMMARY OF THE INVENTION

The present invention relates to an animal model for studying circulating tumour cells constituted of an embryonated bird's egg, preferentially of chicken, comprising human or animal circulating tumour cells (CTC) isolated from a sample of a patient or an animal suffering from cancer, said CTC being grafted at the level of the chorioallantoic membrane (CAM) of an embryonated egg being at a development stage which corresponds to the formation of the CAM at the moment of the graft and equivalent to at least 8 days of development in chickens.

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This embryonated egg is an animal model, and more specifically avian model, for amplifying CTC. It also makes it possible to study the effect of different agents on tumorigenesis to select those which have anti-cancer activity and which may consequently represent efficacious therapies, notably for treating the patient or the animal from which the CTC that have been grafted in the embryonated egg are derived. This then makes it possible to administer to the patient a therapeutic agent tested beforehand on cells of his or her own tumour; thus leading to personalised treatment.

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The present invention also relates to a method for preparing this embryonated bird's egg model, enabling the amplification of human or animal circulating tumour cells (CTC) derived from a sample of a patient or an animal suffering from cancer, said

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method comprising the grafting of CTC isolated from said sample at the level of the chorioallantoic membrane (CAM) of an embryonated bird's egg which is at a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens.

5 The present invention also has for subject matter a method for amplifying human or animal circulating tumour cells (CTC) isolated from a sample of a patient or an animal suffering from cancer, comprising the following steps:

a) preparation of an embryonated egg model in accordance with the invention, by grafting CTC isolated from a sample of a patient or an animal suffering from cancer
10 at the level of the chorioallantoic membrane (CAM) of an embryonated bird's egg which is at a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,
b) collection of tumours that develop from CTC which are amplified, and optionally

c) recovery of CTC from the tumours collected at step b).
15 Preferably, several grafting rounds may be carried out within the scope of this method, and advantageously two grafting rounds, in order to further optimise the amplification of CTC. It thus involves carrying out a secondary graft, on the CAM of a second embryonated egg, of tumours derived from a first graft of CTC on the CAM of a first embryonated egg.

20 The present invention also relates to a method for determining the sensitivity of a patient or an animal suffering from cancer to one or more therapeutic agent(s), characterised in that it comprises:

- amplification of circulating tumour cells (CTC) derived from a sample of said patient or animal suffering from cancer in accordance with the present invention, by grafting
25 CTC isolated from a sample of a patient or an animal suffering from cancer at the level of the chorioallantoic membrane (CAM) of an embryonated bird's egg which is at a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,

- grafting tumours that have developed in the embryo, at the level of the
30 chorioallantoic membrane (CAM) of a new embryonated bird's egg incubated

beforehand up to a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,

- administration of the therapeutic agent(s) in the embryonated egg at least 12 hours after the graft,

- 5 - study of the effect of the therapeutic agent(s) thereby administered on the tumorigenesis of tumours that have developed in this new grafted embryonated egg.

Preferably, this method for determining the sensitivity of a patient or an animal suffering from cancer to one or more therapeutic agent(s) further comprises, after the administration of said agent(s), incubation of the grafted embryonated egg for at
10 least 1 hour, and optionally a step of collection of the tumours that develop from grafted CTC at the end of the incubation of said new grafted embryonated egg.

The present invention further has for subject matter a method for monitoring a patient or an animal suffering from cancer, comprising:

- preparation of a first embryonated bird's egg as described above, with CTC
15 isolated from a sample of said patient or animal at a time T1, and tumorigenesis study of tumours that develop in this first embryonated egg,

- preparation of a second embryonated bird's egg as described above, with CTC isolated from a sample of the same patient or animal at a time T2, and tumorigenesis study of tumours that develop in this second embryonated egg,

- 20 - comparison of the tumorigenesis of tumours that have developed in the first and in the second embryonated egg.

Finally, the present invention has for subject matter a method for screening therapeutic agents intended for the *in vivo* treatment of cancer, comprising:

- preparation of an embryonated egg model according to the method described
25 above, by grafting CTC isolated from a sample of a patient or an animal suffering from cancer at the level of the chorioallantoic membrane (CAM) of an embryonated bird's egg which is at a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,

- administration of one or more candidate agents in the embryonated egg at least 12 hours after the graft,
- study of the effect of the therapeutic agent(s) thereby administered on the tumorigenesis of tumours that have developed in the grafted embryonated egg.

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FIGURE CAPTIONS

Figure 1 represents a diagram of the embryonated egg model with the CTC graft site on the upper CAM.

Figure 2 represents a possible experimental regime for carrying out the invention:

- 10 First grafting round (first group of eggs): CTC isolated from a sample collected from the patient are grafted in one egg per patient. Second grafting round (second group of eggs): the secondary graft enables a new amplification of the tumours collected in the post-graft eggs.

- 15 Figure 3 represents *in ovo* images of the growth of tumours obtained by xenografts on CAM at the first round of amplification of CTC isolated from the blood of patients suffering from lung cancer, breast cancer, or prostate cancer.

Figure 4 represents histological sections of tumours derived from CTC isolated from the blood of patients suffering from breast cancer, grafted on CAM at the first amplification round.

- 20 Figure 5 represents the results of genetic analyses of the human tumour markers TP53 (A) and KRAS (B) on tumours derived from CTC of patients suffering from lung cancer (#SH103 and #CM105), grafted on CAM at the first amplification round.

- 25 Figure 6 represents *in ovo* images of the growth of tumours derived from CTC isolated from the blood of a patient suffering from lung cancer, grafted on CAM at the second amplification round (secondary graft).

Figure 7 represents *in ovo* images of tumours obtained from CTC isolated from the blood of a patient suffering from prostate cancer following the primary (A) and secondary (B) graft, as well as the histological analysis of a section of the tumour

(C) demonstrating the amplification of the CTC grafted *in ovo* after a secondary graft.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention relates to an animal model, in particular an avian model, for studying circulating tumour cells constituted of an embryonated bird's egg comprising human or animal circulating tumour cells (CTC) isolated from a sample of a patient or an animal suffering from cancer, said CTC being grafted at the level of the chorioallantoic membrane (CAM) of an embryonated egg being at a
10 development stage which corresponds to the formation of the CAM and equivalent to at least 8 days of development in chickens at the moment of the graft.

Preferably, the embryonated egg according to the invention is an egg of a bird of the order Galliformes or Struthioniformes. In particular, it is particularly preferred that the egg is an egg of gallinaceans, and notably chicken, quail, turkey, pheasant,
15 peacock, guinea fowl or other farmyard birds. It may also be an ostrich egg. Advantageously, the embryonated egg according to the present invention is a chicken egg (*Gallus gallus*).

Within the scope of the present invention, the term "embryonated egg" designates a fertilised bird's egg in which the embryo can develop under suitable conditions, in particular in an incubator at a temperature of 37°C to 38°C. Under these conditions,
20 the incubation time necessary to end up with the hatching of the egg is 21 days for chicken.

The development stages taught here are defined as a function of the post-fertilisation incubation time of the eggs, in particular the incubation time under
25 suitable conditions such as defined above. "Graft at the level of the CAM" is taken to designate the administration by apposition or injection on the CAM, whether this is the upper CAM or the lower CAM.

The embryonated egg model according to the invention has cells derived from two different organisms or xenografts: the cells of the "host" or "receiver" bird and the
30 CTC grafted in the egg which are derived from a human or animal organism of a species different from that of the "receiver" bird. In a particularly preferred manner,

the CTC grafted in the embryonated bird's egg are human cells. These grafted CTC are then going to develop in the embryo while forming one or more solid tumours and/or while being displaced in the egg.

By definition, the "graft at the level of the CAM" takes place once the CAM has been formed and at a stage equivalent to at least 8 days of development in chickens, under normal and standard growth conditions. If the bird used is a chicken, this stage corresponds to at least 8 days of development. The number of days of development being able to vary from one species to the other, the graft make take place after varying days of development. For example, a development stage of at least 8 days in chickens corresponds to a development stage of at least 6.5 days in quail.

"Sample of patient or animal suffering from cancer" should be understood to mean any sample derived from a human or an animal suffering from cancer and which contains CTC. Preferably, said sample is selected from total blood or from biological fluids capable of containing CTC such as pleural effusion fluid, ascites and cerebrospinal fluid (CSF), and preferably total blood. Thus, the CTC grafted in the embryonated egg may come from any type of cancer producing CTC in the blood or this type of fluid, and in particular metastatic cancers. According to a preferred embodiment of the present invention, the patient or the animal from which the CTC grafted in the embryonated egg are isolated is suffering from at least one cancer selected from lung cancer, prostate cancer, breast cancer, colorectal cancer.

Advantageously, the embryonated egg model according to the invention is a chicken egg in which human CTC have been grafted at the level of the CAM and after at least 8 days of development.

It is understood that the embryo grafted according to the present invention does not have the vocation to hatch and is consequently not intended to create an adult organism. It is for this reason that the embryo grafted according to the present invention is a model intended to receive CTC for the time of their amplification, not extending to hatching, which corresponds to 21 days of development in chickens. Whatever the case, the bird embryo according to the invention will be sacrificed,

according to the ethical rules in force, before hatching and after the grafted CTC will have led to the development of one or more tumours in the egg.

Such a model makes it possible at one and the same time to amplify CTC, notably by the development of tumours from these CTC on which it will be possible to test the anti-cancer activity of different agents, which are either known to have such an activity or candidates to determine if they have such an activity. The comparative efficacy of several therapeutic agents could also be tested, in order to determine the most promising therapy for treating the patient from whom the grafted CTC are derived.

According to a second aspect, the present invention relates to a method for preparing the embryonated bird's egg model described above, enabling the amplification of human or animal circulating tumour cells (CTC) isolated from a sample of a patient or an animal suffering from cancer, said method comprising the grafting of CTC isolated from said sample at the level of the chorioallantoic membrane (CAM) of an embryonated bird's egg that is at a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens.

Those skilled in the art will know how to determine the moment to carry out the grafting of CTC, as a function of the species of bird used, that is to say the minimum number of days of incubation or development of the embryonated egg to arrive at formation of the CAM and at a development stage equivalent to at least 8 days of development in chickens. For example, in chickens, the graft could take place from 8 days of development, and in quail from 6.5 days of development.

According to a preferred embodiment, the embryonated egg has been, prior to the graft, incubated up to a development stage corresponding to the formation of the CAM and equivalent to at least 9 or in an even more preferred manner at least 9.5 days of development in chickens.

The incubations are carried out under suitable conditions, that is to say conditions that enable normal development of the embryonated egg, notably at a temperature comprised between 37°C and 39°C, and preferably 38°C, or even 38.5°C.

The grafting of CTC may be carried out at any place of the CAM, upper or lower, preferably at the level of the upper CAM. Any method well known to those skilled in the art could be used for this graft and, in particular, it is possible to use the grafting technique referenced by Crespo P. & Casar B, 2016.

- 5 According to a particular embodiment, the amount of grafted CTC goes from around 5 to around 5000 CTC, in a preferred manner from around 5 to around 2500 CTC and in an even more preferred manner from around 5 to around 1000 CTC.

According to a preferred embodiment, the CTC used are frozen after isolation from the sample of the patient or animal suffering from cancer and before the graft in the
10 embryonated egg.

For the graft at the level of the CAM, the CTC are isolated from the sample of the patient or animal suffering from cancer, by any one of the methods well known to those skilled in the art. Thus, the CTC are isolated by purifying them from the other cells present in the sample derived from the patient or animal suffering from cancer,
15 and in particular by separating them from immune cells present in the sample.

The methods for isolating CTC may be based on different principles making it possible to separate them from the other constituents of a sample, and consequently to carry out CTC enrichment. A large number of different methods have notably been described by Zheyu Shen *et al.*, 2017. They make it possible to end up with a
20 so-called “negative” enrichment when the objective is to capture the untargeted cells and to elute CTC, or to a so-called “positive” enrichment when the objective is to capture CTC and to elute the untargeted cells of the sample.

Among these methods, it is possible to cite separation methods by filtration, and in particular by vertical filtration such as for example the ISET (Isolation by SizE of Tumor cell) technology sold by the Rarecells SAS Company and notably described
25 by Han Wei Hou *et al.*, 2013.

Certain methods may resort to a marking step, such as the CellSearch method (Kagan M, *et al.*, 2002) which is based on an immuno-selection of CTC using nanoparticles of ferrofluid with antibodies that target a specific epithelial cell

adhesion marker (EpCAM). The CTC are thus separated magnetically from the major part of the other blood cells.

Other methods based on the use of microfluidic systems, which rely on separation as a function of different parameters such as the size, the shape, the density, the deformability of the cells, may also be used. Among these may notably be cited the VTX-1 method such as described by Sollier-Christen *et al.*, 2018, a microfluidic method (CTC-chips) based on the capture of the tumour cells expressing the EpCam molecule using microspots coated with anti-EpCam antibodies, which is notably described by Nagrath S *et al.*, 2007, the RosetteSep technology of the Stemcells Company which uses a mixture of antibodies (CD45, CD66b and glycophorin A) coupled to magnetic beads, targeting red blood cells and white blood cells or instead the ClearCell Fx1 method developed by the Biolidics Company (formerly ClearBridge), which is in particular described by Laget S *et al.*, 2017 and which is particularly advantageous.

According to a particular embodiment, the method for preparing the embryonated bird's egg model according to the invention further comprises a step of incubation of the embryonated egg once grafted for at least 12 hours, preferably at least 24 hours, in an even more preferred manner at least 48 hours after the graft, before being used. Preferably, the embryonated egg once grafted is incubated at the most for 20 days, and in particular up to 18 days, with reference to the stages of development of a chicken embryo.

According to a third aspect, the present invention relates to a method for amplifying human or animal circulating tumour cells (CTC) isolated from a sample of a patient or an animal suffering from cancer, comprising the following steps:

- a) grafting CTC isolated from a sample of a patient or an animal suffering from cancer at the level of the chorioallantoic membrane (CAM) of an embryonated bird's egg which is at a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens, as described above,
- b) collection of tumours that develop from the CTC that are amplified, and optionally
- c) recovery of CTC from the tumours collected at step b).

The inventors have demonstrated, in a surprising manner, that CTC and in particular CTC of human origin may be amplified in an embryonated bird's egg model, which makes it possible to obtain them in sufficient amounts to study them, but also to use them to lead to the identification of novel treatments or to select that which promises to be the most efficacious in an individualised manner.

According to a preferred embodiment of the method for amplifying human or animal circulating tumour cells (CTC) described above, it is possible to graft once again the CTC that will have been amplified by carrying out for example several grafting and collection rounds (the term secondary graft is employed for two grafting rounds), and this is so in order to further improve the amplification of CTC. Thus, the method may comprise the following steps:

a) grafting CTC isolated from a sample of a patient or an animal suffering from cancer at the level of the chorioallantoic membrane (CAM) of an embryonated bird's egg that is at a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,

b) collection of tumours that develop from CTC that are amplified,

b1) grafting the tumours collected at step b) at the level of the chorioallantoic membrane (CAM) of a second embryonated bird's egg incubated beforehand up to a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,

b2) optionally incubation of this second embryonated egg thereby grafted for at least 12 hours,

b3) collection of tumours that develop from the tumours grafted at step b1), and optionally,

c) recovery of CTC from the tumours collected at step b3).

According to a particular embodiment, the method for amplifying human or animal circulating tumour cells (CTC) described above further comprises a step of incubating the embryonated egg once grafted for at least 12 hours, preferably at least 24 hours, in an even more preferred manner at least 48 hours after the graft,

before carrying out the collection of tumours derived from grafted CTC. Preferably, the embryonated egg once grafted is incubated at the most for 20 days, and in particular up to 18 days, with reference to the stages of development of a chicken embryo.

5 The present invention thus also relates to a method for determining the sensitivity of a patient or an animal suffering from cancer to one or more therapeutic agent(s), characterised in that it comprises:

- amplification of CTC isolated from a sample of a patient or an animal suffering from cancer in an embryonated bird's egg, as described above,

10 - grafting the tumours thereby collected at the level of the chorioallantoic membrane (CAM) of a new embryonated bird's egg incubated beforehand up to a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,

15 - administration of the therapeutic agent(s) in the embryonated egg at least 12 hours after the graft,

- study of the effect of the therapeutic agent(s) thereby administered on the tumorigenesis of tumours that have developed in this new grafted embryonated egg.

20 "Therapeutic agent" is taken to designate any chemical or biological molecule or compound, nanostructure, physical methods or combinations thereof having an anti-tumour action, and in particular potentially efficacious for treating the type of cancer that has developed from tumorous cells grafted in the embryonated egg.

25 Such compounds may be chemical molecules such as chemotherapies, biological compounds such as antibodies, therapeutic cells such as CART, physical agents such as irradiations, intercalating agents such as for example Irinotecan, signal pathway inhibitors of tyrosine kinase such as for example Sunitinib, anti-hormonals such as for example Tamoxifen, immunoregulators such as for example Ketruda, membrane receptor inhibitors such as for example Trastuzumab, etc.

Within the scope of the present invention, the terms tumour and cancer are used indifferently, and with the same signification, to define a proliferation of malignant cells. The same is true for the use of the terms antitumoral and anticancer.

5 In particular, since the grafted CTC are isolated from a sample of patient or animal suffering from cancer, the fact of testing several therapeutic agents makes it possible to be able to select that which is the most promising for the treatment of the tumour in this patient. It is thus that, according to a preferred embodiment of the present invention, the embryonated bird's egg grafted with CTC is used to determine the agent that has the best anti-cancer activity among the different agents tested.

10 The embryonated bird's egg grafted with CTC may also be used according to the present invention to test the anti-cancer efficacy of combinations of therapeutic agents compared to the effect obtained with each of the agents tested independently.

15 Within the scope of the use of this embryonated egg, it is also possible to determine, or even to quantify, the toxicity of the therapeutic agent(s) tested, both on the tumours that have developed from grafted CTC and on the embryo as a whole. Consequently, another subject matter of the present invention relates to the use of an embryonated bird's egg grafted with CTC isolated from a sample of a patient or an animal suffering from cancer to quantify the toxicity of one or more therapeutic agent(s) on the tumour and/or on the embryo as a whole.

20 The step of administration of the therapeutic agent(s) in the embryonated egg may be carried out in different ways by techniques well known to those skilled in the art. The administration may notably be carried out by apposition or injection at the level of the CAM, by intra-tumoral injection, by injection into the embryonic or extra-embryonic structures of the egg.

25 The administration of the therapeutic agent(s) is carried out at least 12h after the grafting of CTC, preferably at least 24h, or in an even more preferred manner at least 48h after the graft, that is to say 1 to 2 days after the graft. The therapeutic agent(s) may also be administered according to different regimes in terms of duration, but also number of administrations, such as for example every two days, 30 or every day, or twice a day, or a single injection, and this up to the final day of

incubation of the egg. These choices will be determined as a function of the agent administered.

According to a preferred embodiment, the method for determining the sensitivity of a patient or an animal suffering from cancer to one or more therapeutic agent(s) according to the invention further comprises the incubation of the embryonated egg once grafted for at least 1 hour, after administration of the therapeutic agent(s) in the grafted embryonated egg before studying the effect on tumorigenesis. Advantageously, the incubation is carried out for at least 4 days and at the most 12 days after administration, to correspond to a development stage of the embryo of 21 days maximum, and advantageously 18 days of development. According to a particular embodiment, the method for determining the sensitivity of a patient or an animal suffering from cancer to one or more therapeutic agent(s) according to the invention further comprises the collection of tumours that develop from the grafted CTC at the end of the incubation of said embryonated egg after administration of the therapeutic agent(s) that have been administered, and notably by microdissection. The study of the effect of the therapeutic agent(s) thereby administered on tumorigenesis may take several complementary approaches, in particular after collection of tumours that have developed in the grafted embryonated egg. It may notably comprise the analysis of parameters such as tumour growth, metastatic invasion, angiogenesis, neo-angiogenesis, inflammation and/or tumour immune infiltration, toxicity on the tumour and/or on the embryo as a whole.

The tumours may thus be subjected to analyses for measuring and/or analysing these different parameters, such as tumoral weight and/or volume for studying tumour growth, the expression of different specific markers for studying metastatic invasion such as the amplification of the Alu sequence by quantitative PCR for human metastasis, the number of vessels in the tumour for angiogenesis and neo-angiogenesis, the quantification of interleukins for inflammation and/or the quantification, notably by rtQPCR, of markers such as CD3, CD8, CD4, CD45 and CD56 to assess tumour immune infiltration, weight, and histological analyses to evaluate toxicity on the tumour.

The study of metastatic invasion may be carried out on the lower CAM, easily accessible, but it may also be carried out in any target organ within the embryo, notably as a function of the type of cancer and known data on the associated metastasis phenomenon.

5 Inflammation and/or tumour immune infiltration may notably be studied by the analysis of the expression of different markers, such as CD3 (T lymphocyte membrane marker), CD4 (regulatory T lymphocyte, monocyte and macrophage membrane marker), CD8 (cytotoxic T lymphocyte marker), CD45 (leukocyte membrane marker), CD56 (NK cell marker), etc. Pairs of specific oligonucleotides
10 of these markers will be able to be developed, in order to avoid cross breeding between species.

By extension, it is also possible to monitor inflammation and infiltration of cells of the immune system in sites of metastases.

The combined analysis of all these factors, well known to those skilled in the art,
15 makes it possible to determine the sensitivity of the patient or the animal suffering from cancer from which the grafted CTC are derived to the therapeutic agent(s) administered in the embryo. These parameters notably form an integral part of the decision tree used by clinicians to decide the therapeutic management to adopt in patients suffering from cancer.

20 Within the scope of all the methods according to the invention that comprise the effect of therapeutic agent(s) on tumorigenesis, these parameters are preferentially assessed by comparison after administration of the therapeutic agent(s) in the embryonated egg once grafted compared to those determined in another embryonated egg of the same bird grafted beforehand according to the same
25 method with the same CTC but in which no therapeutic agent has been administered. Similarly, when the effect of several therapeutic agents is studied, the effect will preferentially be assessed by comparison of the parameters after administration of the combination of therapeutic agents in the embryonated egg once grafted compared to those determined in one or more other embryonated
30 egg(s) of the same bird grafted beforehand according to the same method with the

same CTCs but in which each of the therapeutic agents has been administered individually.

5 According to another aspect, the invention also pertains to a method for monitoring a patient or an animal suffering from cancer, comprising:

- preparation of a first embryonated bird's egg as described above with CTC isolated from a sample of said patient or animal at a time T1, and tumorigenesis study of tumours that develop in this first embryonated egg,
- preparation of a second embryonated bird's egg as described above with CTC
10 isolated from a sample of the same patient or animal at a time T2, and tumorigenesis study of tumours that develop in this second embryonated egg,
- comparison of the tumorigenesis of tumours that have developed in the first and in the second embryonated eggs.

15 The present invention also has for subject matter a method for screening therapeutic agents intended for the *in vivo* treatment of cancer, comprising:

- grafting circulating tumour cells isolated from a sample of a patient or an animal suffering from cancer at the level of the CAM of an embryonated bird's egg incubated beforehand up to a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,
 - administration of one or more candidate agents in the embryonated egg, at least
20 12 hours after the graft,
 - study of the effect of the therapeutic agent(s) thereby administered on the tumorigenesis of tumours that have developed in the grafted embryonated egg.
- 25 "Candidate therapeutic agent" is taken to mean a chemical or biologic therapeutic agent such as defined above and capable of having anti-cancer activity, and in particular potentially efficacious for treating the type of cancer that has developed from tumorous cells grafted in the embryonated egg. The screening method according to the present invention makes it possible to determine if a candidate therapeutic agent has anti-cancer activity or not, and if it has anti-metastasis activity.

All the preferences and precisions mentioned above within the scope of the different methods also apply to the monitoring and screening methods according to the present invention.

5 **EXAMPLES**

The invention is illustrated hereafter by the use of an embryonated chicken's egg to amplify human CTC.

Preparation of CTC

10 The CTC grafted in the embryonated eggs are isolated by filtration based on the size of the cells according to the ISET method (Han Wei Hou *et al.*, 2013) or by microfluidic system according to the ClearCell method (Laget S *et al.*, 2017) from blood samples of patients suffering from lung cancer, breast cancer, or prostate cancer in metastasis phase. The CTC thereby obtained are stored frozen, and
15 thawed just before the graft.

Induction of tumours

Fertilised eggs of White Leghorn hens are incubated in the supine position for 9 to 10 days at 37.5°C with a relative humidity of 40%. After this incubation period (E9 or E10), the eggs are prepared to receive the graft. They are opened while
20 preserving the integrity of the CAM, which is lowered by making a small hole through the shell. Then a window of around 1 cm² is cut above the CAM. The CTC (5 to 2500 cells) are grafted by apposition on the CAM, then the eggs are once again incubated at 37.5°C and 40% humidity. After the graft, *in ovo* tumours develop on the upper CAM. The eggs are observed every 48h minimum to monitor the development
25 thereof.

A diagram of an embryonated egg with the CTC graft site on the upper CAM is represented in figure 1. An example of experimental regime is represented in figure 2.

At day 18 of development (E18), the upper part of the CAM is removed, washed in
30 saline phosphate buffer then transferred directly into paraformaldehyde (PFA) for

fixation for 48 hours. The tumours are next carefully extracted from normal CAM tissue.

The tumours collected are next analysed, and notably:

- weighed to study tumour growth (Figure 2, first round);
- 5 - used for histological or genetic analyses (metastases for example) (Figure 2, first round);
- used for a secondary graft in a new batch of embryonated eggs at 9 days of development for amplification (Figure 2, second round). This step may be repeated as many times as necessary (with regular control of the phenotype).

10 Secondary grafts

After 9 days of growth of the grafted CTC, the tumours that have developed are collected and prepared for the carrying out of a secondary graft. The tumour is cut into small pieces and each piece is grafted in the CAM of a new egg in order to obtain a strong amplification of CTC.

15 Analysis of metastases

An analysis of metastases may be carried out either on a portion of the lower CAM (opposite the grafting site) or on embryonic tissues collected at the same time as the tumour and stored in a suitable manner. After extraction of total genomic DNA, the detection of human cells in these samples may be performed by qPCR using
20 specific primers of human Alu sequences (multi-copy sequences well conserved in humans).

Results

The experimental design described above was applied with CTC isolated from the blood of patients suffering from lung cancer (#DA106, #DA107, #PA108, #CB110, #PC111, #VM109, #SH103, #CM105), CTC isolated from the blood of patients
25 suffering from breast cancer (#PS234, #SS226, #UR227, #LP229), CTC isolated from the blood of patients suffering from prostate cancer (#PT319, #BG320).

After 9 days on CAM (E18), all the samples of patient CTC were grafted with success on the CAM and presented a development of tumours. *In ovo* images of the growth of tumours after a first amplification round are grouped together in figure 3.

5 Histological analyses by Haematoxylin/Eosin coloration were carried out on the tumours collected at the first amplification round and derived from CTC isolated from blood of the patient suffering from breast cancer (#PS234), then grafted onto the upper CAM.

10 The results are grouped together in figure 4, which shows the presence of malignant tumour proliferation.

Finally, analyses of genetic mutations were carried out by high speed sequencing according to the “Next Generation Sequencing” (NGS) method referenced in 2017 in the review of Yohe S & Thyagarajan B. with the human tumour markers TP53 (Mogi A & Kuwano H 2011) and KRAS (Gou LY *et al.*, 2015) on genomic DNA
15 extracted from tumours collected at the first amplification round and derived from CTC isolated from the blood of patients suffering from lung cancer. The extraction of DNA was carried out according to the method of the GeneJET FFPE DNA Purification Kit sold by ThermoFisher.

20 The results are grouped together in figure 5, which shows the presence of mutations of the human tumour markers TP53 (Figure 5A) and KRAS (Figure 5B) in two distinct tumours derived from a first graft of CTC of patients suffering from lung cancer (#SH103 and #CM105).

The tumours derived from a first graft of CTC isolated from the blood of a patient suffering from lung cancer were collected at E18, cut up then once again grafted
25 onto the CAM of a second group of embryonated eggs. The *in ovo* images of growth of tumours collected at E18 after a second amplification round are grouped together in figure 6 (A and B correspond to *in ovo* images of tumours on CAM derived from two distinct secondary grafts).

30 The tumours derived from a first graft of CTC isolated from the blood of a patient suffering from prostate cancer (ClearCell Fx1 microfluidic method) were collected at

E18, cut up then once again grafted on the CAM of a second group of embryonated eggs. The *in ovo* images of growth of tumours are grouped together in figure 7. Image A represents the tumour obtained *in ovo* from CTC following the primary graft. The two sites of tumour development are shown by arrows. Image B corresponds
5 to that of an *in ovo* tumour on CAM derived from a secondary graft, produced from the tumour shown in A. Image C corresponds to the histological analysis of a section of the tumour represented in B, showing on the one hand the tissue of the CAM, and on the other hand tumoral tissue.

10 **Conclusions**

The results described above show that the use of an avian embryonated egg model enables the *in ovo* amplification of CTC isolated from samples of patients, in a quicker and less expensive manner compared to the murine model of the prior art. The present invention thus makes it possible to constitute rapidly biobanks of CTC,
15 which may next be used for efficacy tests of anti-cancer therapies, for monitoring and screening novel agents capable of being able to give rise to efficacious and personalised treatments.

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CLAIMS

1. Embryonated bird's egg model comprising human or animal circulating tumour cells (CTC) isolated from a sample of a patient or an animal suffering from cancer, said CTC being grafted at the level of the chorioallantoic membrane (CAM) of an embryonated egg being at a development stage which corresponds to the formation of the CAM and equivalent to at least 8 days of development in chickens at the moment of the graft.

2. Model according to claim 1, characterised in that the embryonated egg is at a development stage of at the most 20 days.

3. Method for preparing an embryonated bird's egg model according to any one of claims 1 or 2 enabling the amplification of human or animal circulating tumour cells (CTC) isolated from a sample of a patient or an animal suffering from cancer, said method comprising the following steps:

- grafting CTC isolated from said sample at the level of the chorioallantoic membrane (CAM) of an embryonated bird's egg which is at a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,
- optionally incubation of the embryonated egg thereby grafted for at least 12 hours.

4. Method for amplifying human or animal circulating tumour cells (CTC) isolated from a sample of a patient or an animal suffering from cancer, comprising the following steps:

- a) preparation of an embryonated egg model using the method according to claim 3,

- b) collection of tumours that develop from CTC that are amplified,
- c) optionally, recovery of CTC from the tumours collected at step b).

5. Method for amplifying human or animal circulating tumour cells (CTC)
5 according to claim 4, characterised in that it comprises the following steps:

- a) preparation of a first embryonated egg model using the method according to claim 3,
- b) collection of tumours that develop from CTC that are amplified,
 - 10 b1) grafting the tumours collected at step b) at the level of the chorioallantoic membrane (CAM) of a second embryonated bird's egg incubated beforehand up to a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,
 - b2) optionally, incubation of this second embryonated egg thereby grafted for at least 12 hours,
 - 15 b3) collection of tumours that develop from the tumours grafted at step b1), and optionally,
 - c) recovery of CTC from the tumours collected at step b3).

6. Method for determining the sensitivity of a patient or an animal suffering from
20 cancer to one or more therapeutic agent(s), characterised in that it comprises:

- amplification of circulating tumour cells (CTC) isolated from a sample of said patient or animal suffering from cancer according to the method such as defined in any one of claims 4 to 5,
- grafting the tumours thereby collected at the level of the chorioallantoic
25 membrane (CAM) of a new embryonated bird's egg incubated beforehand up to a development stage corresponding to the formation of the CAM and equivalent to at least 8 days of development in chickens,

- administration of the therapeutic agent(s) in the embryonated egg at least 12 hours after the graft,
- study of the effect of the therapeutic agent(s) thereby administered on tumorigenesis of the collected tumours that have developed in this new grafted embryonated egg.

5

7. Method for determining the sensitivity of a patient or an animal suffering from cancer to one or more therapeutic agent(s) according to claim 6, characterised in that the study of tumorigenesis comprises the assessment or the measurement of different parameters such as tumour growth, metastatic invasion, angiogenesis, neo-angiogenesis, inflammation and/or tumour immune infiltration, toxicity on the tumour and/or on the embryo as a whole.

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8. Method for monitoring a patient or an animal suffering from cancer, comprising:

15

- preparation of a first embryonated bird's egg according to claim 3 with CTC isolated from a sample of said patient or animal at a time T1, and tumorigenesis study of the tumours that develop in this first embryonated egg,

20

- preparation of a second embryonated bird's egg according to claim 3 with CTC isolated from a sample of the same patient or animal at a time T2, and tumorigenesis study of the tumours that develop in this second embryonated egg,

- comparison of the tumorigenesis of tumours that have developed in the first and in the second embryonated eggs.

25

9. Method for screening therapeutic agents intended for the *in vivo* treatment of cancer, comprising:

- preparation of an embryonated egg model using the method according to claim 3,
- administration of one or more candidate agents in the embryonated egg at least 12 hours after the graft,
- 5 - study of the effect of the therapeutic agent(s) thereby administered on the tumorigenesis of tumours that have developed in the grafted embryonated egg.