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(54) **Title:** METHOD OF PREDICTING CHEMOTHERAPEUTIC RESPONSIVENESS OF CANCER

(57) **Abstract:** Disclosed is a method of predicting clinical tumor outcome by providing gene expression from a tumor sample. The method utilizes a novel genetic screen to identify genes that contribute to chemotherapeutic responsiveness, using formalin fixed paraffin embedded clinical samples of epithelial cancer, specifically serous ovarian cancer. The method is useful in predicting tumor responsiveness to chemotherapeutics, including alkylating agents, cisplatin, antimetabolites, plant alkaloids, and antitumor antibiotics. A microarray screen showed formalin fixed paraffin embedded samples can identify genes related to chemotherapeutic response with 86% efficiency.

METHOD OF PREDICTING CHEMOTHERAPEUTIC RESPONSIVENESS OF CANCER

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to currently pending U.S. Provisional Patent Application No. 60/981,963, entitled "Method of Predicting Chemotherapeutic Responsiveness", filed on October 23, 2007, the contents of which are herein incorporated by reference.

FIELD OF INVENTION

This invention relates to cancer diagnosis methods. Specifically, the invention is a method of determining the response of a cancer to chemotherapy using gene expressions of formalin-fixed paraffin embedded tissue samples.

STATEMENT OF GOVERNMENT INTEREST

This invention was made with Government support under Grant No. DAMD17-02-2-0051 awarded by the Department of Defense. The Government has certain rights in the invention

BACKGROUND OF THE INVENTION

A cornerstone of personalized cancer care will be the ability to predict how an individual patient will respond to a therapeutic intervention. Recent reports suggest that gene expression profiles have the potential to discriminate between patients who will and will not respond to specific chemotherapeutic agents. However, most existing gene expression predictive signatures have been developed and tested in fresh-frozen (FF) tissues and their utility in formalin fixed paraffin embedded (FFPE) samples, commonly encountered in clinical practice, is unknown.

Formalin fixation and wax embedding is a universal tissue processing procedure, allowing samples to be cut into thin sections(i.e. a few microns) stored at room temperatures indefinitely. Formalin-fixed paraffin-embedded (FFPE) archival clinical specimens are invaluable in discovery of prognostic and therapeutic targets for diseases such as cancer. Acquisition of appropriate clinical samples remains a fundamental problem in diagnosis. Tissue biopsies are difficult to obtain and therefore are too valuable to be used in global diagnosis development in most instances. A vast archive of tissue samples exists for every conceivable condition as formalin-fixed (FF) and paraffin-embedded (PE) samples. FFPE samples are prepared by incubating the tissue in a buffered formalin solution of 3.7% (w/v)

formaldehyde and 10–15% methanol, forming intra-and inter-molecular covalent crosslinks between proteins, RNA and DNA (Fox, C.H., et al., Formaldehyde fixation. *J. Histochem. Cytochem.* 1985; 33:845-53; Kunkel, G.R., et al., Contact-site cross-linking agents. *Mol. Cell Biochem.* 1981; 34:3-13). Afterwards, the samples are embedded with paraffin, which enables FFPE samples have been used to diagnose and stage tumors and evaluate protein expression by immunohistochemistry (IHC) and *in situ* hybridization.

Throughout a century of use, numerous archival paraffin-embedded tissue banks have been established worldwide. These tissue banks are invaluable resources of tissues for translational studies of cancer and various other diseases. Accessibility of macromolecules in the samples is a critical issue, as FFPE samples are traditionally limited to IHC.

Recent developments in extraction methodologies have opened FFPE samples to new analyses, like MS. An antigen retrieval (AR) technique, by boiling FFPE samples in water was shown to enhance IHC by circumventing the formalin fixation, and is now the typical approach for IHC staining of FFPE samples (Shi et al. 1991). Recently, AR and proteinase K/SDS treatment has been shown useful in extracting nucleic acids (Hood et al. 2006; Dubeau et al. 1986). These techniques rely on either strong heating of FFPE samples or enzyme digestion.

Proteomic studies of FFPE samples have been severely limited due to the formaldehyde-induced crosslinking, which renders proteins insoluble and unsuitable for biochemical extraction and analysis. For example, crosslinking prevents extraction of proteins from FFPE samples for use in protein analysis, such as Western blots. The advances in FFPE processing techniques have yet to overcome these obstacles, since many proteins are still undetectable (Crockett, D., et al., Identification of proteins from formalin-fixed paraffin-embedded cells by LC-MS/MS. *Lab. Invest.*, 2005; 85:1405-1415).

A predictor for ovarian cancer response to platinum-based therapy is needed for use with stable patient samples, such as formalin fixed paraffin embedded samples.

SUMMARY OF THE INVENTION

Disclosed is a method of predicting clinical tumor outcome by providing gene expression from a tumor sample (Shedden, K., et al., Gene expression–based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. Nat. Med. 14(8):822-7. (2008)). The gene expression may be obtained from any number of means known in the art, including without limitation, Polymerase Chain Reaction, ChIp, gene array, microarrays or quantitative-Polymerase Chain Reaction (Q-PCR), and reverse transcriptase Polymerase Chain Reaction (rt-PCR). The present method utilizes a novel genetic screen to identify genes that contribute

to chemotherapeutic responsiveness, using formalin fixed paraffin embedded clinical samples. A microarray screen showed formalin fixed paraffin embedded samples can identify genes related to chemotherapeutic response with 86% efficiency. At least one threshold value is defined for classifying the gene expression levels. The extracted biological material is selected from the group consisting of DNA, RNA, protein, derivatives thereof, and fragments thereof. As used herein, derivatives refer to processed variants of DNA, RNA, or proteins, which includes, without limiting the scope of the invention, transcripts. In certain embodiments, the disclosed method uses RNA to determine gene expression. Gene expression levels are determined from the biological material in the clinical sample and compared to the gene expression of the clinical sample with a gene expression of known clinical outcome, indicative of tumor outcome. In some embodiments, the gene expression of known clinical outcome was constructed by correlating gene expression levels to clinical outcome, classifying gene expression levels by clinical outcome into gene expression groups, comparing variance between the gene expression groups, and fitting a statistical model to the gene expression groups. In specific embodiments, the statistical model is a binary regression model.

As such, disclosed is a method of predicting clinical tumor prognosis by extracting a biological material from a formalin-fixed paraffin-embedded clinical sample. Also disclosed is a method of predicting clinical tumor responsiveness to chemotherapeutic treatment from a formalin-fixed paraffin-embedded clinical sample. A method is also disclosed for predicting clinical tumor responsiveness to chemotherapeutic treatment for ovarian from a formalin-fixed paraffin-embedded clinical sample. In some embodiments, the disclosed methods are useful for predicting, without limiting the scope of the invention, tumor prognosis of epithelial cancer, and in specific embodiments serous ovarian cancer. In more specific embodiments, the method is useful for predicting tumor responsiveness to chemotherapeutic treatment selected from the group consisting of alkylating agent, antimetabolite, plant alkaloid, and antitumor antibiotic. In further embodiments, the method is useful for predicting tumor responsiveness to cisplatin.

The disclosed methods utilize Polymerase Chain Reaction, ChIp, gene array, microarrays, reverse transcriptase Polymerase Chain Reaction, and quantitative-Polymerase Chain Reaction to determine gene expression in some embodiments. In certain embodiments, the methods further generated a probeset list and tested the plurality of gene expressions in the clinical sample for gene expression using the probeset list. In these embodiments, the probeset list was generated by providing a first probeset and testing the first probeset against gene expression data for a tumor cell with known chemotherapeutic outcome, wherein the gene expression data is compared to the known chemotherapeutic outcome. In some

embodiments of the disclosed methods, the chemotherapeutic treatment is selected from the group consisting of alkylating agent, antimetabolite, plant alkaloid, and antitumor antibiotic, and in more specific embodiments, the chemotherapeutic is cisplatin.

BRIEF DESCRIPTION OF THE DRAWINGS

For a fuller understanding of the invention, reference should be made to the following detailed description, taken in connection with the accompanying drawings, in which:

The Figure is a datasheet of the 115 probe set.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

"Patient" is used to describe an animal, preferably a human, to whom treatment is administered, including prophylactic treatment with the compositions of the present invention.

Disclosed is a tumor prognosis predictor based on gene expression signatures of cancer cells. Gene expression data is used to identify a patient's tumor response chemotherapeutic intervention. The invention uses cumulative expression information from a series of genes involved in the regulation of the cell cycle and the mitotic process. This information is then used to categorize tumor samples based on the chemotherapeutic responsiveness using a mathematical model and gene expression data derived from microarrays or quantitative-Polymerase Chain Reaction (Q-PCR) data.

183 FFPE advanced stage (III/IV) serous ovarian cancers were identified, obtained during primary surgical cytoreduction from patients who went on to receive platinum-based chemotherapy. Archival formalin-fixed, paraffin-embedded tissue samples were obtained from the processed tissue as follows. Immediately after excision from the patients, samples were routinely fixed in 10% neutral buffered formalin [average period of fixation was 24 hr at room temperature (RT)]. Fixed tissues were processed routinely through dehydration in graded ethanol, clearing in xylene, and embedding in paraffin blocks using automatic processing and embedding equipment.

RNA was extracted using the High Pure RNA Paraffin Kit (Rosche Diagnostics GmbH, Mannheim, Germany). Briefly, each paraffin block was cut at 5-10 µm and xylene added for 5 min. Absolute ethanol was added to the sample and spun at 12,000 x g, the supernatant removed and pellet allowed to dry. The samples were lysed using a 10%SDS/ Proteinase K buffer overnight at 55°C. Proteins were precipitated using an ethanol/buffer solution and proteins collected as provided.

The extracted RNA was amplified using the WE-Ovation System (NuGen). Total RNA was measured and subjected to reverse transcriptase (RT) PCR using DNA/RNA chimeric primers. The RNA was fragmented with RNase H, forming priming sites for PCR. Amplification of the product formed double stranded cDNA and the resultant product was purified by centrifugation as provided. After amplification, the samples were loaded on an Affymetrix 133 plus 2.0 GeneChip array and analyzed.

All hybridizations were carried out at 45°C for 16–17 h with mixing on a rotisserie at 60 rpm. Following hybridization, the solutions were removed and the arrays were rinsed with 1x MES. The arrays were washed and stained using the GeneChip Fluidics station protocol EukGE_WS2, which consists of 10 cycles of 2 mixes per cycle with non-stringent wash buffer (6x SSPE, 0.01% Tween 20) at 25°C followed by 4 cycles of 15 mixes per cycle with stringent wash buffer (100 mM MES, 0.1 M Na+, and 0.01% Tween 20) at 50°C. The probe arrays were stained for 10 min in streptavidin-phycoerythrin solution (SAPE) [1x MES solution, 10 µg/ml SAPE (Molecular Probes, Eugene, OR), and 2 µg/µl acetylated BSA (Invitrogen)] at 25°C, then washed for 10 cycles of 4 mixes per cycle at 25°C. The probe arrays were treated for 10 min with an antibody solution [1x MES solution, 2 µg/µl acetylated BSA, 0.1 µg/µl normal goat IgG (Sigma Chemical, St. Louis, MO), 3 µg/µl biotinylated goat-anti-streptavidin antibody, (Vector Laboratories, Burlingame, CA)] at 25°C followed by a second staining for 10 min in SAPE at 25°C. The final wash was 15 cycles of 4 mixes per cycle at 30°C with non-stringent wash buffer. The probe arrays were then scanned once at 1.56 µm resolution using the Affymetrix GeneChip Scanner 3000 or at 3 µm resolution using the Affymetrix GeneChip Scanner 2500.

Arrays were visually scanned for any defects or scanning artifacts that might compromise the final results. 87 of the younger samples were divided into a training set (n=44), with the remaining samples held out as an external validation datasets containing similar microarray data test (n=43).

Training set GeneChip results were subjected to ANOVA and Binary regression analysis to develop and test gene expression profiles associated with ovarian cancer platinum-responsiveness.

The binary regression model was provided by:

$$y_i \mid \pi_i = Ber(\pi_i), \qquad \pi_i = \Pr(y_i = 1) = F(\mathbf{x}_i'\beta), \tag{01}$$

(Collet, 1994), where y_i=1 if the response of interest is observed for the i^{th} individual, p_i is the probability the i^{th} individual is responsive, β is the K vector of unknown parameters,

$\mathbf{x}_i^t = (x_{i1}, \ldots, x_{iK})$ the K vector of known covariates associated to the i^{th} individual and F any transformation for 0 and 1. Thus,

$$F(\mathbf{x}_i^t\beta) = \begin{cases} \exp(\mathbf{x}_i^t\beta)/[1+\exp(\mathbf{x}_i^t\beta)], & (logistic) \\ \Phi(\mathbf{x}_i^t\beta), & (probit) \\ 1-\exp[-\exp(\mathbf{x}_i^t\beta)]. & (complementary\ log\text{-}log) \end{cases} \quad (02)$$

Thereby providing the formula,

$$p(\mathbf{y} \mid \beta) = \prod_{i=1}^{n} [F(\mathbf{x}_i^t\beta)]^{y_i} [1 - F(\mathbf{x}_i^t\beta)]^{(1-y_i)}. \quad (03)$$

The data underwent Bayesian analysis, making

$$p(\beta \mid \mathbf{y}) \propto p(\beta) \prod_{i=1}^{n} [F(\mathbf{x}_i^t\beta)]^{y_i} [1 - F(\mathbf{x}_i^t\beta)]^{(1-y_i)}. \quad (04)$$

The 115 probe set predictor, shown in The Figure, was used to test the patient samples. The Predictor correctly identified 27/33 (82%) platinum complete responders (CR) and 10/11 (91%) incomplete responders (IR) for an overall accuracy of 37/44 (84%) in leave-one-out cross validation. In the external validation set, the 115 probe set predictor correctly identified 26/32 (81%) CR and 9/11 (82%) IR samples, with an overall accuracy of 35/43 (81%).

Microarray expression analysis of FFPE samples identified genes that influence ovarian cancer platinum-responsiveness and is useful in predicting chemoresponsiveness in a chemo-predictive assay with clinical utility.

In the preceding specification, all documents, acts, or information disclosed does not constitute an admission that the document, act, or information of any combination thereof was publicly available, known to the public, part of the general knowledge in the art, or was known to be relevant to solve any problem at the time of priority.

The disclosures of all publications cited above are expressly incorporated herein by reference, each in its entirety, to the same extent as if each were incorporated by reference individually.

While there has been described and illustrated specific embodiments of a tumor prognosis prediction method, it will be apparent to those skilled in the art that variations and modifications are possible without deviating from the broad spirit and principle of the present

invention. It is also to be understood that the following claims are intended to cover all of the generic and specific features of the invention herein described, and all statements of the scope of the invention which, as a matter of language, might be said to fall therebetween. Now that the invention has been described,

What is claimed is:

1. A method of predicting clinical tumor prognosis comprising the steps of:

extracting a biological material from a formalin-fixed paraffin-embedded clinical sample, wherein the biological material is selected from the group consisting of DNA, RNA, protein, derivatives thereof, and fragments thereof;

establishing a plurality of gene expressions in the clinical sample from the biological material; and

comparing the gene expression of the clinical sample with a gene expression of known clinical outcome; wherein the gene expression of known clinical outcome is indicative of tumor outcome.

2. The method of claim 1, wherein the clinical tumor outcome is predicted for epithelial cancers.

3. The method of claim 2, wherein the clinical tumor outcome is predicted for serous ovarian cancer.

4. The method of claim 1, wherein the tumor prognosis is predictive for tumor responsiveness to chemotherapeutic treatment selected from the group consisting of alkylating agent, antimetabolite, plant alkaloid, and antitumor antibiotic.

5. The method of claim 4, wherein the chemotherapeutic is cisplatin.

6. The method of claim 1, wherein the biological material is RNA.

7. The method of claim 1, wherein the gene expression was established using a method selected from the group consisting of Polymerase Chain Reaction, ChIp, gene array, microarrays, reverse transcriptase Polymerase Chain Reaction, and quantitative-Polymerase Chain Reaction.

8. The method of claim 1, wherein the gene expression of known clinical outcome was constructed further comprising the steps of:

correlating gene expression levels to clinical outcome;

classifying gene expression levels by clinical outcome into gene expression groups;

comparing variance between the gene expression groups; and

fitting a statistical model to the gene expression groups.

9. The method of claim 8, wherein the statistical model is a binary regression model.

10. The method of claim 1, further comprising the steps of:

generating a probeset list, comprising:

providing a first probeset;

testing the first probeset against gene expression data for a tumor cell with known chemotherapeutic outcome, wherein the gene expression data is compared to the known chemotherapeutic outcome; and

testing the plurality of gene expressions in the clinical sample for gene expression using the probeset list.

11. A method of predicting clinical tumor responsiveness to chemotherapeutic treatment comprising the steps of:

extracting a biological material from a formalin-fixed paraffin-embedded clinical sample, wherein the biological material is selected from the group consisting of DNA, RNA, protein, derivatives thereof, and fragments thereof;

establishing a plurality of gene expressions in the clinical sample from the biological material; and

comparing the gene expression of the clinical sample with a gene expression of known clinical outcome; wherein the gene expression of known clinical outcome is indicative of tumor outcome.

12. The method of claim 11, wherein the clinical tumor outcome is predicted for epithelial cancers.

13. The method of claim 12, wherein the clinical tumor outcome is predicted for serous ovarian cancer.

14. The method of claim 11, wherein the chemotherapeutic treatment is selected from the group consisting of alkylating agent, antimetabolite, plant alkaloid, and antitumor antibiotic.

15. The method of claim 14, wherein the chemotherapeutic is cisplatin.

16. The method of claim 11, wherein the biological material is RNA.

17. The method of claim 11, wherein the gene expression was established using a method selected from the group consisting of Polymerase Chain Reaction, ChIp, gene array, microarrays, reverse transcriptase Polymerase Chain Reaction, and quantitative-Polymerase Chain Reaction.

18. The method of claim 11, wherein the gene expression of known clinical outcome was constructed further comprising the steps of:

correlating gene expression levels to clinical outcome;

classifying gene expression levels by clinical outcome into gene expression groups;

comparing variance between the gene expression groups; and

fitting a statistical model to the gene expression groups.

19. The method of claim 18, wherein the statistical model is a binary regression model.

20. The method of claim 11, further comprising:

generating a probeset list, comprising:

providing a first probeset;

testing the first probeset against gene expression data for a tumor cell with known chemotherapeutic outcome, wherein the gene expression data is compared to the known chemotherapeutic outcome; and

testing the plurality of gene expressions in the clinical sample for gene expression using the probeset list.

21. A method of predicting clinical tumor responsiveness to chemotherapeutic treatment for ovarian cancer comprising the steps of:

extracting a biological material from a formalin-fixed paraffin-embedded clinical sample, wherein the biological material is selected from the group consisting of DNA, RNA, protein, derivatives thereof, and fragments thereof;

establishing a plurality of gene expressions in the clinical sample from the biological material;

comparing the gene expression of the clinical sample with a gene expression of known clinical outcome; wherein the gene expression of known clinical outcome is indicative of tumor outcome, further comprising the steps of:

correlating gene expression levels to clinical outcome;

classifying gene expression levels by clinical outcome into gene expression groups;

comparing variance between the gene expression groups; and

fitting a statistical model to the gene expression groups.

22. The method of claim 21, wherein the clinical tumor outcome is predicted for serous ovarian cancer.

23. The method of claim 21, wherein the chemotherapeutic treatment is selected from the group consisting of alkylating agent, antimetabolite, plant alkaloid, and antitumor antibiotic.

24. The method of claim 23, wherein the chemotherapeutic is cisplatin.

25. The method of claim 21, wherein the biological material is RNA.

26. The method of claim 21, wherein the gene expression was established using a method selected from the group consisting of Polymerase Chain Reaction, ChIp, gene array, microarrays, reverse transcriptase Polymerase Chain Reaction, and quantitative-Polymerase Chain Reaction.

27. The method of claim 21, wherein the statistical model is a binary regression model.

28. The method of claim 21, further comprising:

generating a probeset list, comprising:

providing a first probeset;

testing the first probeset against gene expression data for a tumor cell with known chemotherapeutic outcome, wherein the gene expression data is compared to the known chemotherapeutic outcome; and

testing the plurality of gene expressions in the clinical sample for gene expression using the probeset list.

weight 115 probes	115 probes
0.088126	241797_at
0.084467	1560342_at
0.082193	235411_at
0.074582	209423_s_at
0.071843	237237_at
0.07029	230853_at
-0.069006	202129_s_at
0.06688	239504_at
0.06232	214759_at
0.060026	236207_at
0.059493	239784_at
0.059488	225355_at
0.05915	208530_s_at
0.058835	212170_at
0.058817	225062_at
0.056911	213478_at
0.05626	209615_s_at
-0.056133	227005_at
-0.055894	204243_at
0.055654	208777_s_at
-0.055216	229528_at
0.053579	226793_at
0.050939	1555841_at
-0.050938	235200_at
-0.05035	202613_at
0.049972	239136_at
0.049901	1566144_at
0.049658	234883_x_at
-0.048841	238938_at
0.048201	1557918_s_at
0.047647	224015_s_at
0.047051	233348_at
0.046457	219452_at
0.045805	226846_at
-0.04505	230855_at
0.044107	234955_at
-0.044045	209081_s_at
0.043847	243752_s_at
0.043579	207550_at
0.042888	231885_at
0.041608	222347_at
0.041285	1559559_at
0.041079	202266_at
-0.040419	220913_at
-0.039943	227043_at
0.039046	213872_at

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-0.036289	213482_at
-0.036221	40829_at
-0.036075	1566145_s_at
-0.035973	224878_at
-0.035451	209055_s_at
0.034685	218345_at
0.034374	234317_s_at
-0.034023	208580_x_at
-0.033858	203144_s_at
-0.033197	238127_at
0.031736	243637_at
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-0.030783	232379_at
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-0.030286	201007_at
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0.025661	1553212_at
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weight 115 probes	115 probes
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0.015263	219341_at
0.012863	220684_at
-0.011934	1570318_at
-0.011661	1560932_at
-0.00868	203346_s_at
-0.005512	217659_at
0.004061	215955_x_at

The Figure

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US08/80939

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/574 (2008.04)
USPC - 435/7.23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - G01N 33/574 (2008.01)
USPC - 435/7.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/6, 4

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest (PGPB, USPT, EPAB, JPAB), Google Patents, Google Scholar - cisplatin, paraffin-embedded, tumor, formalin, antitumor antibiotic, plant alkaloid, antimetabolite, antitumor antibiotic, chemotherapeutic, serous, ma, ovarian, epithelial, ma, statistical, binary regression model, probeset, gene expression

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2006/0154250 A1 (MORRIS et al.) 13 July 2006 (13.07.2006), para [0004], [0013], [0031], [0034], [0037], [0040], [0076], [0077], [0092], [0163], [0165], [0170], [0178], [0183], [0224], [0264], [0267], [0269], [0272], [0273], [0275], [0282], [0284], [0298], [0299], [0311], [0316]-[0321]	1-3, 6, 7, 11-13, 16, 17 --- 4, 5, 8-10, 14, 15, 18-28
Y	US 2007/0172844 A1 (LANCASTER et al.) 26 July 2007 (26.07.2007), Tables 4, 5, para [0015], [0016] [0028] [0029], [0052], [0055], [0064], [0071], [0072], [0088], [0101], [0111], [0121], [0122], [0125], [0135], [0141]-[0143], [0161], [0165], [0166], [0176], [0197], [0198], [0202], [0203], [0206], [0210], [0217], [0224], [0225], [0227], [0232], [0234], [0237], [0240], [0243], [0244], [0246], [0263], [0266], [0269], [0288], [0291], [0293]	4, 5, 8-10, 14, 15, 18-28

☐ Further documents are listed in the continuation of Box C. ☐

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Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2008 (20.12.2008)	05 JAN 2009
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