Title: DIAGNOSIS OF METASTASES IN HNSCC TUMOURS

Abstract: The invention relates to the detection or prediction of metastases of head and neck squamous cell carcinoma (HNSCC) with the use of gene expression profiles. A gene signature has been identified which is able to detect or predict the occurrence of these metastases better than current clinical methods. Part of the invention are micro-arrays comprising this signature and methods for performing the detection and/or prediction.
Title: Diagnosis of metastases in HNSCC tumours

FIELD OF THE INVENTION

The invention relates to the field of tumour diagnosis, in particular to predict the existence of metastases of a tumour, more in particular to the detection of lymph node metastases of head and neck squamous cell carcinoma (HNSCC) especially those that arise in the oral cavity and oropharynx.

STATE OF THE PRIOR ART


Metastasis is the process whereby cancers spread to distinct sites in the body. It is the principal cause of death in individuals suffering from cancer. For some tumor types, the earliest detectable sign of metastasis is the presence of malignant cells in lymph nodes close to the site of the primary tumour. Early detection of local lymph node metastases is currently pivotal for appropriate treatment of many types of cancer. However, because of difficulties in detecting lymph node metastases reliably, many patients currently receive inappropriate treatment.

Most patients with HNSCC, especially those in the oral cavity or oropharynx have the primary tumour removed. Treatment of clinically diagnosed lymph node metastasis positive patients (N+) involves the additional surgical removal of a significant portion of the neck, including all five local lymph node levels: radical

Clinical diagnosis of N0 lymph node status is even less accurate. Histological examination of electively operated clinically diagnosed N0 patients reveals that about one-third have positive neck nodes (Jones, A.S. et al., (1993) Eur. Arch. Otorhinolaryngol. 250, 446-449). Different strategies exist for neck treatment of N0 diagnosed patients (Pillsbury, H.C., et al., (1997) Laryngoscope 107, 1294-1315). One is the so-called "watch and wait" strategy by which N0 diagnosed patients do not undergo any neck dissection. The involves the risk of fatality by allowing overlooked metastases to develop and spread further. Since the prevalence of false-negative predictions is very high, most clinics perform neck surgery for all diagnosed N0 patients. In this case most often a supra-omohyoidal neck dissection (SOHND) is performed, removing the three upper lymph node levels (Robbins, K.T. et al., supra).

This treatment is less appropriate than an RND for those N+ patients falsely diagnosed as N0 and, moreover, completely unnecessary for all patients correctly diagnosed as N0. Although SOHND is less rigorous than RND, the treatment cause disfigurement, long-term discomfort, pain and can lead to additional complications such as shoulder disability (e.g. Short, S.O. et al., (1984) Am. J. Surg. 148, 478-482). Both treatments strategies result in over- or undertreatment due to limitations in detecting lymph node metastasis reliably.


For HNSCC such expression signatures are starting to be uncovered (Chung, C.H. et al., (2004) Cancer Cell 5, 489-500), but as yet without independent validation for reliability and clinical outcome.

Thus, there is still a large need for a reliable diagnostic tool on basis of expression of genes with which a reliable and accurate prediction can be established for the presence or occurrence of lymph node metastasis in HNSCC.

SUMMARY OF THE INVENTION

The invention now provides a nucleotide array of maximal 50 nucleotide sequences, preferably maximal 100 nucleotide sequences, more preferably maximal 1000 nucleotide sequences, for the detection of metastasis in head and neck squamous cell cancer (HNSCC) comprising at least 1 of the elements of Table 5, more preferably 2 of the elements, more preferably 3 of the elements, more preferably 4 of the elements., more preferably 5 of the elements, more preferably 6 of the elements%, more preferably 7 of the elements, more preferably 8 of the elements, more preferably 9 of the elements, more preferably 10 of the elements and most preferably at least 20 of the elements. Alternatively, a nucleotide array for the detection of metastasis in HNSCC has 50 or more of the elements of the genes listed in Table 4.

Further provided is a method to establish reference and control gene expression profiles of patients having had metastasis after HNSCC (N+ group) or no metastasis after HNSCC (N0 group) by analysing the gene expression from a tumour biopsy sample of each patient, or from pooled samples of each group of patients, on an array according to the invention.

Another embodiment of the invention is a method to predict the presence or risk of occurrence of lymph node metastasis of a HNSCC patient. comprising:

a. taking a biopsy sample from the tumour of the patient;
b. isolating the nucleic acid from the biopsy sample;
c. analyse the gene expression profile of said nucleic acid by assaying it with a nucleotide array according to the invention;
d. classifying the expression profile as N+ or N0 by determining whether the expression profile would match the expression profile of a group of HNSCC patients known to have developed metastasis.

Preferably, the biopsy samples in the above methods are fresh biopsy samples.

A preferred embodiment for the method to predict the presence or risk of metastasis is a method, wherein the analysis of the gene expression profile comprises:

a. hybridising the nucleic acid form the biopsy sample with the nucleotide array according to the invention;

b. determining the amount of hybridisation of each of the elements of the nucleotide array relative to the amount of hybridisation of each element with a reference sample, said step optionally involving a normalisation step;

c. determining for each element of the array whether the expression of the corresponding gene in the biopsy sample is more or less than the expression of the corresponding gene in the reference sample.

Preferably, the expression profile is classified as N+ (high risk of metastasis) or N0 (low or no risk of metastasis) according to the steps of:

a. determining the collective correlation of the classifier/predictor genes or elements present in the expression profile with the average N+ or N0 profile from primary tumors with previously established N-status; and

b. determining the predictive threshold based on the correlation threshold from primary tumors with previously established N-status

In another preferred embodiment the method is a method, wherein the gene expression profile of a group of HNSCC patients known to have developed metastasis is the expression profile contained in the dataset E-UMCU-11, available in the public microarray database ArrayExpress (http://www.ebi.ac.uk/arrayexpress/).

Calculation of the correlation as performed in the above methods is preferably done using the cosine correlation method.

Normalization of the expression profile is preferably achieved by correcting the expression data for experimental variations with the help of expression data of a control gene or element which is not affected by the tumour state, preferably by calculating the ratio of the expression data of each gene or element in the array of claim 1 or 2 with the expression of a control gene or element or the mean of a pool of control genes or elements.
LEGENDS TO THE FIGURES

Figure 1. A predictor for HNSCC lymph node metastasis. (a) Expression profiles of the 102 predictor genes on the 82 primary tumor training set (middle). The predictor genes are clustered based on their similarities across the 82 tumors (Pearson around zero correlation, centroid clustering). Tumors are rank-ordered according to their correlation with the average N0 expression profile (left). The solid line represents the threshold for optimal overall accuracy. Tumors above the threshold show an expression profile that indicates that the patient is free of lymph node metastasis. In the right panel the patient’s histological N-status, including the 3 year follow-up period, and the clinical diagnosis are shown (black indicates post-operative histological N+, white indicates post-operative histological N0, dark grey indicates clinical N+ and light grey indicates clinical N0 assessment). The asterix indicates a patient that developed lymph node metastasis post-treatment. (b) Expression profiles and tumor correlations from 6 training tumors samples (circles) and their technical replicates (squares). (c) as (a) only for a independent validation set of 22 primary HNSCC tumors. The threshold is set according to the optimal threshold established with the latter half of the training set (Fig. 2d).

Figure 2. Long-term tissue storage results in loss of predictive accuracy. (a) The mean correlation with the average no-metastasis profile and the standard deviation range for N0 patients (blue) and N+ patients (red) in the training set are shown for each year of surgery. (b) The N0 (blue), N+ (red) and overall (green) predictive accuracies increase from 40–45% for samples from 1996, to 89–100% for samples from 2000. (c,d) Correlation data from tumors with longer (c) or shorter (d) storage time. The predictor correctly predicts 22 of the 38 and 38 of the 44 samples, respectively.

Figure 3. The predictor outperforms current clinical diagnosis on the validation set. (a) Predictive accuracies (PA) of current clinical diagnosis (blue) and the predictor
(red) on the validation set. Error bars are based on the standard error for predictive accuracy. The predictor has a N0 PA of 100%, N+ PA of 77%, and overall PA of 86%. Clinical diagnosis has a N0 PA of 67%, N+ PA of 71%, and overall PA of 68%. (b,c) Treatment accuracy for the validation set, based on current clinical diagnosis (b) or if based on predictor outcome (c). Completely appropriate treatment is shown in green and under- or overtreatment in red. Current diagnosis resulted in 23% of patients receiving appropriate treatment (50% of N+ receiving an RND). Predictor based treatment would result in 86% of patients receiving appropriate treatment (75% of N0 that no longer receive any neck dissection and 100% of N+ receiving a RND).

Figure 4. Study design and procedures overview. a, RNA was isolated from 2-3 tumor sections, followed by mRNA amplification and fluorescent labeling. After hybridization, scanned images were quantified and the data was normalized. Duplicates of each tumor were averaged and a predictor was designed using the differentially expressed genes. Quality control monitoring occurred after total RNA isolation, cRNA synthesis, labeling, scanning and normalization. b, The training experiment design involved 82 primary HNSCC tumors, compared in duplicate dye-swap against a common reference pool containing equal amounts of cRNA from each tumor. Nine reference pool self-self comparisons were generated in parallel, to establish an error-model for technical variation. c, The predictor was designed using a double loop training-validation protocol.

Figure 5. The predictive outcome of different signatures is stable. Predictive correlation outcome of 66 tumor samples using a multiple training approach. A thousand different molecular signatures comprising 50 (A), 100 (B) or 200 (C) genes were used to predict each sample approximately 100 times. Samples from patients without metastasis are colored blue (top line in the graph), samples from patients with lymph node metastasis are colored red (bottom line in the graph). The shaded area represents the 95%-confidence interval for the sample predictions.

DETAILED DESCRIPTION OF THE INVENTION
The inventors herein show that it is possible to give a more accurate prediction of the presence of lymph node metastasis of HNSCC than currently possible, by measuring mRNA expression of a concise set of genes (the predictor signature). It appeared possible to give an accurate prediction on basis of a set of 102 genes listed in Table I. It appeared that half of these genes have not been directly associated with tumorigenesis or metastasis before. Besides expected epithelial marker genes, interesting categories include genes (putatively) coding for extracellular matrix components, genes involved in cell adhesion including three members of the plakin family of cytolinkers and the enzyme transglutaminase 3, which play a role in maintaining tissue integrity; cell death genes; cell growth and maintenance genes and genes encoding hydrolyzing activities including proteins involved in degradation of the extracellular matrix (uPA and PAI-1) and a metalloproteinase. Another feature of the metastasis signature is that there is more down-regulation associated with metastasis (two thirds) than up-regulation. It is likely that this involves stromal and immune-regulatory components (Pollard, J.W. (2004) Nat. Rev. Cancer 4, 71-78; Chambers, A.F. et al. (2002) Nat. Rev. Cancer 2, 563-572). Many of the predictor genes belong to this categories, strengthening the argument for profiling bulk tumour tissue rather than laser-dissected regions densely populated with tumour cells.

It is shown herein that a diagnosis/prediction of the presence of metastases can be given using expression data of a set of only five genes from this large set of 102 genes. Table 2 indicates 15 of the genes which rank high in predictive value and which can especially be used to give a diagnosis or prediction of metastasis in HNSCC. Of course, accuracy of prediction will increase when more then five, preferably all 15 and even more preferably all 102 genes will be used on an array for gene expression analysis for the diagnostic/predictive signature.

Gene expression analysis is preferably done using a micro-array. The techniques for measuring and comparing gene expression on micro-arrays is well established within the art. It should be understood that it is not necessary to have the full length nucleotides encoding the above mentioned genes on said array: a stretch of nucleotides which is sufficient to establish unique hybridisation with the RNA expressed from said genes in the tumour cells can be used. Such a stretch of
nucleotides is hereinafter referred to as 'element'. Preferably for the specific use of gene expression analysis for the current invention (i.e. with relation to detection of the presence of or the risk for metastases of HNSCC) such an array need not contain a large number of (different) genes or elements. It would be sufficient for the array to contain the necessary genes, as discussed above, and, preferably, some control genes, as will be discussed below. The array, which can be used for the analysis of the invention thus does not need to contain more than 1000 genes or elements, preferably not more than 500 genes or elements, more preferably not more than 200 genes or elements and most preferably from about 50 to about 150 genes or elements.

To investigate a gene expression profile the array should be subjected to hybridisation with target polynucleotide molecules from a clinically relevant source, in this case e.g. a person with HNSCC. Therefore, preferably a fresh frozen (within 1 hour from surgical removal), liquid nitrogen (at least -80 °C) stored tumour sample needs to be available. Said target polynucleotide molecules should be expressed RNA or a nucleic acid derived therefrom (e.g., cDNA or amplified RNA derived from cDNA that incorporates an RNA polymerase promoter). If the target molecules consist of RNA, it may be total cellular RNA, poly(A)^+ messenger RNA (mRNA) or fraction thereof, cytoplasmic mRNA, or RNA transcribed from cDNA (cRNA). Methods for preparing total and poly(A)^+ messenger RNA are well known in the art, and are described e.g. in Sambrook *et al.*, (1989) Molecular Cloning: A Laboratory Manual (2nd Ed.) Vols. 1-3, Cold Spring Harbor, New York. In one embodiment, RNA is extracted from cells using guanidinium thiocyanate lysis followed by CsCl centrifugation (Chrigwin *et al.*, (1979) Biochem. 18:5294-5299). In another embodiment, total RNA is extracted using a silica-gel based column, commercially available examples of which include RNeasy (Qiagen, Valencia, CA, USA) and StrataPrep (Stratagene, La Jolla, CA, USA). Poly(A)^+ messenger RNA can be selected, e.g. by selection with oligo-dT cellulose or, alternatively, by oligo-dT primed reverse transcription of total cellular RNA. In another embodiment, the polynucleotide molecules analyzed by the invention comprise cDNA, or PCR products of amplified RNA or cDNA.

Preferably, the target polynucleotides are detectably labelled at one or more nucleotides. Any method known in the art may be used to detectably label the nucleotides. Preferably, this labelling incorporates the label uniformly along the length of the polynucleotide and is carried out at a high degree of efficiency. One
embodiment for this labelling uses oligo-dT primed reverse transcription to incorporate the label; however, conventional methods hereof are biased toward generating 3' end fragments. Thus, in this embodiment, random primers (e.g. 9-mers) are used in reverse transcription to uniformly incorporate labelled nucleotides over the full length of the target polynucleotides. Alternatively, random primers may be used in conjunction with PCR methods or T7 promoter-based in vitro transcription methods in order to amplify the target polynucleotides.

In a preferred embodiment, the detectable label is a luminescent label. For example, fluorescent labels, bioluminescent labels, chemiluminescent labels and colorimetric labels may be used. In a highly preferred embodiment, the label is a fluorescent label, such as a Cy5 or Cy3, fluorescein, a phosphor, a rhodamine, or a polymethylene dye or derivative. In another embodiment, the detectable label is a radiolabeled nucleotide.

The array may be any nucleotide array which represents five or more of the genes of Table 2 or Table 1. To indicate the difference with the existing very large arrays of e.g. Affymetrix, the dedicated arrays of the present invention should preferably comprise no more than 50, or 100, or 250 or, alternatively 500 or 1000 genes altogether. Presence of other genes on the array is allowable and the expression data from such other genes need not necessarily be considered for the present application. The methods of the invention can be applied on the above mentioned dedicated arrays, but can also be performed on arrays that are commercially available (e.g. from Agilent US; Affymetrix Inc, CA, USA; and others). It is also possible to work with self-made arrays by spotting or synthesizing nucleotides which are known to selectively hybridise to the target genes on a surface. Methods to prepare such arrays are well within the skill of the artisan. The microarrays can comprise cDNA, but can also comprise short oligonucleotides (Affimatrix and Nimblegen) or long oligonucleotides which are synthesized in situ (Agilent); in another embodiment the arrays comprise long oligonucleotides and are self-made by spotting.

Nucleic acid hybridisation and wash conditions are chosen so that the target polynucleotide molecules specifically hybridize to the complementary polynucleotide sequences of the array, preferably to a specific array site, wherein its complementary DNA is located. Optimal hybridisation conditions will depend on the type (e.g., RNA or DNA) of the target nucleotides and array. General parameters for
specific (i.e., stringent) conditions of hybridisation are described in Sambrook et al. (supra). Typical hybridisation conditions for cDNA microarrays are hybridisation in 5 X SSC plus 0.2% SDS at 65 °C four hours, followed by washes at 25 °C in low stringency wash buffer (1 X SSC plus 0.2% SDS), followed by 10 minutes at 25 °C in higher stringency wash buffer (0.1 X SSC plus 0.2% SDS).

When fluorescence labelled probes are used, the fluorescence emissions at each site of the microarray may be detected by scanning confocal laser microscopy. In one embodiment, the arrays is scanned with a laser fluorescent scanner with a computer controlled X-Y stage and a microscope objective. Fluorescent laser scanning devices are described in e.g. Schena et al. (1996) Genome Res. 6:639-645. Signals are recorded and, in a preferred embodiment, analysed by computer using a 12 or 16 bit analog to digital board. In one embodiment the scanned image is despeckled using a graphics program (e.g., Hijaak Graphics Suite) and then analysed using an image gridding program that creates a spreadsheet of the average hybridisation at each wavelength at each site.

Not all of the genes are evenly contributing to the discriminating effect. As is shown in Table 1, the genes differ in significant expression. Although the statistical data presented in the Examples are calculated with all of the 102 genetic elements of Table 1, it is submitted that a good distinction between the two groups of patients and therewith a good diagnosing/predicting ability of the signature gene set can also be achieved with only a part of the elements of Table 1. At least 5 (5%) of the elements of Table 1 are included in the analysis, more preferably 20%, more preferably 40%, more preferably 60%, more preferably 80%, more preferably 90% and most preferably all of the elements. It would be advisable not to randomly choose the elements, but to pick the most discriminating genes in this list. Table 2 gives an overview of the top 15 genes out of the 102 genes of table 1, of which at least 5, more preferably at least 6, more preferably at least 7, more preferably at least 8, more preferably at least 9, more preferably at least 10, more preferably at least 11, more preferably at least 12, more preferably at least 13, more preferably at least 14, and most preferably all 15 can be used for making up the signature with which the microarray analysis is performed.

It furthermore has been found that a more comprehensive set of predicting genes can be compiled by repeatedly calculating a predictive signature via a multiple training approach (similar to Michiels, S. et al., Lancet 365:488-492, 2005). In this

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study (see Examples) it appeared that from the originally more than 2000 differentially expressed genes only 825 (Table 3) had a predictive character, and that for these a subgroup of 179 (Table 4) genes was used in more than half of the signatures. From this group again a supergroup of 61 genes (Table 5) could be distinguished which was predominantly used to discriminate between N+ and N0. It will be understood that preferably an array would comprise at least three, but preferably five, more preferably 10, even more preferably 25 and most preferably 61 of the genes of Table 6. However, it also appeared possible to classify on basis of genes, which did not occur in Table 5, but in such cases many genes are required to achieve an acceptable prediction. Thus, an array could also comprise at least 10, preferably 25, more preferably 50, and most preferably 100 of the genes of Table 5.

As indicated above, various combinations of these genes can be used for determining the presence of lymph node metastases in several ways.

On dual channel DNA microarrays this is performed by determining the expression level ratios of the genes in the primary tumour sample versus expression of the same genes in reference material. The reference material can be derived from a pool of total RNA or amplified mRNA from a set of HNSCC primary tumours with established lymph node metastasis characteristics. The individual gene expression ratios contribute towards the expression ratio signature of a sample. The degree of correlation of a sample’s signature with the signatures of samples with known metastatic status (preferably calculated by the cosine correlation (Jones, W. P., & Furnas, G. W. (1987). Pictures of Relevance: A Geometric Analysis of Similarity Measures. Journal of the American Society for Information Science, 36 (6), 420-442) as, e.g., provided by the Genesis software; http://genome.tugraz.at/Software/Genesis/Description.html) is used to predict the metastatic state of the unknown sample. The correlation threshold for predicting the metastatic state is based on the optimal threshold for discriminating between the metastatic states of the samples with known metastatic states, which can easily be determined by a person skilled in the art.

Other measurements of absolute expression and expression ratios of these genes can also be used. Reference material can be derived from other sources than a pool of samples with known metastatic states. Preferably, however, samples with
known metastatic states are still required to determine the correlation threshold for determining the metastatic status.

Expression ratios can also be derived from single channel microarray experiments, using as a reference so-called housekeeping genes (i.e. with stable expression across many different samples) or a collection of housekeeping genes or any collection of genes or features with stable expression. Again here it is preferred to use samples with known metastatic states to determine the correlation threshold for determining the metastatic status.

Gene expression measurements and the derived ratios can also be obtained by (quantitative) reverse transcription PCR or any other assay for gene expression, using as a reference any gene or collection of genes that have stable expression across many samples. In a specific embodiment of this application of the invention, samples with known metastatic states are still required to determine the correlation threshold for determining the metastatic status.

In the absence of tumour samples with known metastatic states for calibration of the prediction, the genes or various combinations of the (expression analysis of the) genes can still be used to predict the metastatic state. In these embodiments of the invention an absolute or relative measurement of gene expression is determined for example using single or dual channel DNA microarrays, or by other methods such as (quantitative) reverse transcription PCR. Increased expression of the genes in table 1 or 2 with a positive N+ correlation will hereby contribute positively towards prediction of the N+ status and negatively towards prediction of the N0 status. Conversely, increased expression of the genes in table 1 or 2 with a negative N+ correlation will contribute positively towards N0 prediction and negatively towards N+ prediction. Increased expression in both cases indicates an increase relative to a suitable marker gene or feature, set of genes or features or collectively in relation to each other.

However, a person skilled in the art is able to obtain the reference data that have been produced in the below example, since this data is available as dataset E-UMCU-11 from the public micro-array database ArrayExpress.
(http://www.ebi.ac.uk/arrayexpress/). When desiring to predict or determine the presence of metastases for a certain patient, the practitioner should take a biopsy from that patient, isolate the RNA and determine the expression of at least 5 of the elements of Table 1. To normalize these expression data with respect to the data of the reference set E-UMCU-11, it is possible to correct the data for variations with the help of expression data of a control gene or element which is not affected by the tumour state (such as a housekeeping gene), which is present in the reference set E-UMCU-11 and should also be available on the array that has been used to determine the expression profile of the patient to be assessed. In stead of one control gene or element, also the mean value of a poll of control genes or elements can be taken. This correction can, for instance, be done by subtracting the expression level of the control gene(s)/element(s) from the expression levels of each of the tested genes/elements. Preferably, the ratio for every tested gene(s) with respect to the control gene(s) is calculated for both the patient's expression profile as well as for the expression data of the reference set.

With these figures, the correlation with the mean value of the N0 values of the reference set should be calculated. If this correlation is negative (i.e. a value below zero) it can be concluded that the patient is N+ (i.e. having or prone to develop metastases). Conversely, the correlation can be calculated with respect to the mean value of the N+ values of the reference set. Then a negative correlation indicates a match with the N0 group.

Further enablement for a diagnosis/prediction of cancer metastasis on basis of gene expression analyses can be found in WO 03/010337, indicating that methods as have been generally described above are well within the skills of the practitioners in the art.

Example

Data accessibility
MIAME\(^1\) compliant data in MAGE-ML\(^2\) format as well as complete descriptions of protocols, microarrays and clinical parameters have been submitted to the public microarray database ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with the following accession numbers: Microarray layout, A-UMCU-3; HNSCC tumour data, E-UMCU-11; Protocols for sectioning of tumour material, P-UMCU-18; RNA isolation, P-UMCU-19; DNase treatment, P-UMCU-20; mRNA amplification, P-UMCU-21; generating reference pool, P-UMCU-26; cRNA labeling, P-UMCU-22; hybridization and washing of slides, P-UMCU-23 and P-UMCU-24; scanning of slides, P-UMCU-25; Image analysis, P-UMCU-11

**Tumor samples**

For the training set, 92 samples were randomly taken from a collection of primary tumours surgically removed between 1996 and 2000 and that fulfilled the following criteria: biopsy-proven HNSCC in the oropharynx and oral cavity; no previous malignancies in the head and neck region; tumour sections contained more than 50% tumour cells. Of these 92 tumours, 82 passed total RNA and cRNA quality control (QC) and were included in this study. For the validation set, 27 tumours were randomly taken from the same collection of tumours, surgically removed between 2000 and March 2001, and that fulfilled the same selection criteria. Of these, 22 passed total RNA and cRNA QC and were included in this study. The diagnostic procedures for clinical staging of cervical lymph nodes was performed according to the Netherlands national guidelines for oral cavity and oropharynx carcinomas, by clinical examination (palpation) of the neck region, followed by bilateral ultrasound examination, computed tomography (CT) and/or magnetic resonance imaging (MRI).

Suspected nodes were subjected to aspiration cytology. In this way, patients were pre-operatively classified as either N0 or N+, the latter in the case of aspirates yielding metastatic tumour cells. Only in the case of obvious neck involvement, as shown by huge swelling, were the patients classified as N+ without additional efforts to prove the presence of metastasis.

Surgery was aimed at complete tumour removal. With regard to the neck, in the case of clinical N0 only a SOHND was performed\(^3\). In cases clinically classified as N+ a RND was performed including all five lymph node levels\(^3\). Postoperative irradiation was administered in accordance with current practice and depending on margin
status, tumour growth features, number of positive nodes and extracapsular growth. In practice, 36 out of 60 clinically assessed N0 patients and 38 out of 43 clinically assessed N+ patients received radiation therapy. This treatment as well as additional clinical information is presented in Supplemental data 2 (for accessibility, see above).

After surgery, patients were periodically checked for development of neck metastasis, and patients initially classified as N0 but showing positive nodes in their surgical specimen or developing neck nodes within a time span of 3 years after surgery without having another head and neck cancer that could be responsible for this metastasis, were retrospectively added to the N+ patient group. Less than 5% of patients with HNSCC in the oral cavity or oropharynx subsequently develop metastasis after treatment\textsuperscript{4,5}. Here, for the training and validation cohorts, one patient subsequently developed positive neck nodes after surgery. Three years is to be considered as a reliable time period, since at least 80% of the recurrences are known to take place in the first two years after surgery (Takes, R.P. \textit{et al.} (2001) J Pathol \textbf{194}, 298–302 ; Jones, K.R., \textit{et al.}, (1992) Arch. Otolaryngol. Head Neck Surg. \textbf{118}, 483–485).

Fresh tumour tissue was taken from the surgical specimen, snap-frozen in liquid nitrogen immediately after surgical removal and stored at –80°C. Frozen sections were cut for RNA isolation and immediately transferred to a RNAlater solution (Ambion). A haematoxylin and eosin stained section was prepared for tumour percentage assessment. Only samples with at least 50 percent tumour cells were used. For a small number of samples the tumour percentage was increased by removing areas with no tumour cells.

\textbf{RNA isolation}

Total RNA was isolated from 2-3 sections (20 µm) with TRIzol reagent (Invitrogen), followed by a purification using the RNeasy mini-kit (Qiagen) and a DNase treatment using the Qiagen DNA-free kit. The yield and quality of total RNA was checked by spectrophotometry and by the Agilent 2100 Bioanalyser (Agilent). Total RNA quality control criteria were in accordance with the Tumour Analysis Best Practices Working Group\textsuperscript{6}, discarding samples with no clear 18S and 28S ribosomal bands. We also removed samples that had a yield lower than 500 ng total RNA or showed mycoplasma contamination.
cRNA synthesis and labeling
mRNA was amplified by *in vitro* transcription using T7 RNA polymerase on 1 μg of total RNA. First a double stranded cDNA template was generated including the T7 promoter. Next, this template was used for *in vitro* transcription with the T7 megascript kit (Ambion) to generate cRNA. During the *in vitro* transcription, 5-(3-aminoallyl)-UTP (Ambion) was incorporated into the single-stranded cRNA. The yield and quality of the cRNA was analyzed by spectrophotometry and by the Agilent 2100 Bioanalyzer. Samples with a yield less than 5000 ng or with small cRNA fragments (median less than 500 bp) were not used.

Cy3 or Cy5 fluorophores (Amersham) were coupled to 500 ng of cRNA. After coupling, free dye molecules were removed using Clontech ChromoSpin-30 columns (Clontech). The yield and label incorporation (5-7%) of the cy-labeled cRNA was checked using spectrophotometry. Before hybridization, 300 ng of cy-labeled cRNA from one tumor was mixed with an equal amount of reverse color cy-labeled material from the reference sample.

Microarray production
The Human Array-Ready Oligo set (version 2.0) was purchased from Qiagen and printed on Corning UltraGAPS slides as described elsewhere. The microarrays contained 70-mer oligonucleotides representing 21,329 genes as well as 3871 additional features for control purposes.

Microarray hybridization
Before use, the microarray slides were treated with sodium-borohydrate solution to reduce auto-fluorescence in the cy3-channel. The labelled cRNA targets were hybridized on the microarray for 10 hours at 42 °C using the Ventana Discovery Hybridization Station in combination with the ChipMap-80 Kit (Ventana Europe). After hybridization the slides were manually washed and scanned in the Agilent G2565AA DNA Microarray Scanner (100% laser power, 30% PMT).

Pre-processing of expression data
The scanned images were quantified and background corrected using Image 4.0 software (Biodiscovery). The expression data was normalized for dye and print-tip biases using a Lowess per print-tip normalization algorithm applied in the statistical package R. Following normalization, variance stabilization (VSN) was applied to stabilize variance in the intensity data. Both duplicate dye-swap hybridizations of each tumor were averaged and for each gene a tumor-reference ratio was calculated. Reference versus reference hybridizations were used to build a gene error model for technical variation. Nine reference self-self comparisons were performed in dye-swap (18 hybridizations), resulting in nine reference ratios for each gene on the microarray. These nine reference ratios yield an estimate of the technical variation for each gene. To test whether a gene in a tumor samples shows differential expression, a Student's t-test was applied on the tumor ratio and the corresponding nine reference ratios (technical variation). The calculated p-values for differential expression were used to select those genes that show differential expression in the tumor samples.

**Supervised classification**

A classifier was constructed to distinguish between N0 and N+ patients. Of the 21,329 genes on the microarray, 6221 were excluded based on aberrant signal and spot morphology in one of the 164 hybridizations. From these remaining 15,108 genes, only genes that were significantly different from the reference in at least 31 tumours were selected based on the error model for technical variation (p<0.01). This resulted in a set of 1,986 genes. For these genes the signal-to-noise-ratio (SNR) was computed and employed to rank the genes (top ranked genes being genes that are best suited to distinguish the outcome classes). The optimal gene set to employ in the classifier (a nearest mean classifier similar to the classifier employed in), was determined by gradually expanding the gene set starting from the highest ranked gene. At each expansion round the nearest mean classifier was trained on a training set and tested on a test set. The performance on the test set served as a quality measure of the gene set. The performance was measured as the average of the false positive (N0 classified as N+) and false negative (N+ classified as N0) rates of the test samples. Initially the performance increases as the set is expanded. The expansion of the gene set is terminated when the performance deteriorates, i.e. when the optimal performance is
reached. The steps of ranking the genes and training and testing the classifier are performed in a 10-fold cross-validation procedure. The output of this procedure is an optimal number of top-ranked genes and a trained classifier. To ensure independent validation, this process of optimizing the set of genes and training the classifier is wrapped in a second 3-fold cross-validation loop. This entails that the optimization of the gene set and the training of the classifier is performed on 2/3 of the data, while the classifier is validated on 1/3 of the data. Since this 1/3 of the data is never involved in any of the gene selection and training steps, this ensures completely independent validation of the classifier, which avoids selection bias\textsuperscript{14,15} and therefore results in a reliable performance estimate. This double-loop procedure determined 102 genes to form the final diagnostic classifier. This classifier was trained on the complete set of 82 samples by recalculating the signal-to-noise ratio for all genes and subsequently selecting the top 102 genes. The predictor was trained using the 102 selected genes and the 82 training samples. A decision threshold for this classifier was fixed such that the highest overall predictive accuracy for both N0 and N+ tumours was reached.

**Statistics**

Odds ratios (OR) were calculated by fitting a logistic regression model on the prediction outcome of the validation set. The predictor had an infinitive OR since no false negative prediction was made. To get an estimate of the OR for the predictor, one false negative was artificially introduced resulting in a predictor OR of 30 \( (p=0.006) \) and a clinical OR of 4.2 \( (p=0.15) \). The standard error for predictive accuracy (Fig. 3a) includes the predictions made on the latter half of the training set.

**Selection of predictive genes**

A multiple training approach was used to identify a complete set of predictive genes, based on the 66 tumor samples from 1998 to 2001. The tumor samples were randomly divided into a training set and test set using a 10-fold cross validation procedure. Based on the training set, \( p \)-values were calculated for all 3064 differentially expressed genes based on the difference in expression between N+ and N0 tumor samples (Student's T-test). The set of genes with lowest \( p \)-values (i.e. most-predictive) was used for prediction of the test samples by calculating the correlation

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with the average N+ and average N0 training profile and, based on these correlations, classifying the test samples as N0 or N+. Repeating this resampling procedure a thousand times resulted in multiple predictions for each tumor sample, based on the different predictive gene sets. This approach was repeated three times to determine 1000 predictive gene sets consisting of 50 genes, 1000 gene sets of 100 genes and 1000 gene sets of 200 genes. All gene sets had predictive value (Figure 1). Genes selected at least once are listed in Table 3. This consists of 825 genes with predictive power for detection or prediction of metastasis in head and neck squamous cell carcinoma. Small and large sets of genes from this list can be used for prediction (Figure 5).

Genes selected more frequently, that is present in more than 50% of the 200 gene set predictors are listed in Table 4. This consists of 179 genes with strongest predictive power for detection or prediction of metastasis in head and neck squamous cell carcinoma. Small and large sets of genes from this list can be used for prediction. Genes selected most frequently (more than 90%) are listed in Table 5. This consists of 51 genes with the highest predictive power. Small and large sets of genes from this list can be used for prediction. This list consists of genes, most/all of which have never before been associated with prediction of metastasis in tumors, especially metastasis in head-neck squamous cell carcinoma.

References


Table 1. Complete list of the 102 HNSCC predictor genes

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CATB_HUMAN  CTSB  NM_001908  297939  0.2069354  Cathepsin B 4
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NGAP_HUMA  RASAL2  NM_004841  227806  0.1877348  RAS protein activator like 2 6
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0.1965940 full length insert cDNA
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clone EUROIMAGE
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APA1_HUMAN APOA1  NM_000039
93194 0.1945643 Apolipoprotein A-I
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IKBA_HUMAN NFKBIA  NM_020529
81328 0.1943403 Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
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S51G_HUMAN SEC61G  NM_014302
9950 0.1938544 Sec61 gamma
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Q8TB66  NIFK  NM_032390
142838 0.1923585 Nucleolar protein interacting with the FHA domain of pK-67
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FXLA_HUMAN PCCX2  AB031230
199009 - Protein containing 0.1922701 CXXC domain 2
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HSAC005389  PRO2086  NM_014111
60082 - PRO2086 protein
0.1918323
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TGDS_HUMAN TDPGD  NM_014305
12393 0.1912078 DTDP-D-glucose 4,6-dehydratase
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Q9NYL1  PTOV1  NM_017432
19555 0.1894273 Prostate tumor over expressed gene 1
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RET7_HUMAN RBP7  NM_052960
292718 0.1890260 Retinoid binding protein 7
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Q9H5V9  FLJ22965  NM_022101
248572 0.1865584 Hypothetical protein
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O94911  ABCA8  NM_007168
38095 - ATP-binding cassette, 0.1860282 sub-family A (ABC1), 9

AAH50307  MORC  NM_014429
278908 - Microchondia homolog 0.185651 (mouse)
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TR1B_HUMAN TNFRSF1B  NM_001066
256278 0.1856088 Tumor necrosis factor receptor superfamily, member 1B
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HS71_HUMAN HSPA1A  NM_005345
8997 0.1832944 Heat shock 70kD protein 1A
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Q9C086  PAPA-1  NM_031288
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0.1817337
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HSAC000871  0AK024270  4094 0.1741561 Homo sapiens cDNA FLJ14208 fis, clone NT2RP3003264
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**TABLE 4**

List of 179 genes with strong predictive value
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<td>NM_018357 Hypothetical protein FLJ11196</td>
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<td>CTSK</td>
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SUBSTITUTE SHEET (RULE 26)
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Claims

1. A nucleotide array of maximal 50 nucleotide sequences, preferably maximal 100 nucleotide sequences, more preferably maximal 1000 nucleotide sequences, for the detection of metastasis in head and neck squamous cell cancer (HNSCC) comprising at least 1 of the elements of Table 5, more preferably 2 of the elements, more preferably 3 of the elements, more preferably 4 of the elements, more preferably 5 of the elements, more preferably 6 of the elements, more preferably 7 of the elements, more preferably 8 of the elements, more preferably 9 of the elements, more preferably 10 of the elements and most preferably at least 20 of the elements.

2. A nucleotide array for the detection of metastasis in HNSCC having 50 or more of the elements of the genes listed in Table 4.

3. A method to establish reference and control gene expression profiles of patients having had metastasis after HNSCC (N+ group) or no metastasis after HNSCC (N0 group) by analysing the gene expression from a tumour biopsy sample of each patient, or from pooled samples of each group of patients, on an array comprising the elements mentioned in claim 1 or 2, or an array according to claim 1 or 2.

4. A method to predict the presence or risk on occurrence of lymph node metastasis of a HNSCC patient. comprising:
   a. taking a biopsy sample from the tumour of the patient;
   b. isolating the nucleic acid from the biopsy sample;
   c. analyse the gene expression profile of said nucleic acid by assaying it with a nucleotide array comprising the elements mentioned in claim 1 or 2, or an array according to claim 1 or 2;
   d. classifying the expression profile as N+ or N0 by determining whether the expression profile would match the expression profile of a group of HNSCC patients known to have developed metastasis.

5. A method according to claim 3 or 4, where the biopsy sample is a fresh biopsy sample.

6. A method according to claim 4, wherein the analysis of the gene expression profile comprises:
a. hybridising the nucleic acid form the biopsy sample with the nucleotide array comprising the elements mentioned in claim 1 or 2, or an array according to claim 1 or 2;
b. determining the amount of hybridisation of each of the elements of the nucleotide array relative to the amount of hybridisation of each element with a reference sample, said step optionally involving a normalisation step;
c. determining for each element of the array whether the expression of the corresponding gene in the biopsy sample is more or less than the expression of the corresponding gene in the reference sample.

7. A method according to claim 4 or 6, wherein the expression profile is classified as N+ (high risk of metastasis) or N0 (low or no risk of metastasis) according to the steps of:

a. determining the collective correlation of the classifier/predictor genes or elements present in the expression profile with the average N+ or N0 profile from primary tumors with previously established N-status; and
b. determining the predictive threshold based on the correlation threshold from primary tumors with previously established N-status

8. A method according to any of claims 4, 6 or 7, using the data contained in the E-UMCU-11 dataset in the public microarray database ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), which contains all relevant gene expression measurements for patients with established metastatic status.

9. A method according to claims 7 or 8, wherein the correlation is determined using the cosine correlation method.

10. A method according to any of claims 6 to 9, wherein the normalization of the expression profile is achieved by correcting the expression data for experimental variations with the help of expression data of a control gene or element which is not affected by the tumour state, preferably by calculating the ratio of the expression data of each gene or element in the array of claim 1 or 2 with the expression of a control gene or element or the mean of a pool of control genes or elements.
Figure 1
Figure 2
Figure 3
Study design and procedures overview. a, RNA was isolated from 2-3 tumor sections, followed by mRNA amplification and fluorescent labeling. After hybridization, scanned images were quantified and the data was normalized. Duplicates of each tumor were averaged and a predictor was designed using the differentially expressed genes. Quality control monitoring occurred after total RNA isolation, cRNA synthesis, labeling, scanning and normalization. b, The training experiment design involved 82 primary HNSCC tumors, compared in duplicate dye-swap against a common reference pool containing equal amounts of cRNA from each tumor. Nine reference pool self-self comparisons were generated in parallel, to establish an error-model for technical variation. c, The predictor was designed using a double loop training-validation protocol. See methods section and Supplementary Information for details.
Figure 5

A  n=50  

B  n=100  

C  n=200  

[Graph showing correlation with samples for n=50, n=100, n=200]