INTRODUCTION

Microarrays technique finds ever wider application both in oncological research and in other fields of science (1). Laryngeal carcinoma, like the majority of head and neck cancers, is the malignancy with well-determined cancerogenic factors. Thus, the analysis of its transcriptome is an interesting model from the point of view of cancer molecular biology. At the same time, the clinical course of laryngeal carcinoma is difficult to predict on the basis of the so far recognised clinical and histopathological criteria. A more thorough recognition of the molecular mechanisms in laryngeal carcinoma may allow for development of new prognostic or predictive markers or treatment methods (2, 3). Simultaneously, the analysis of genes and proteins that exhibit changed expression in laryngeal carcinoma shall facilitate the diagnostic process in the cases, where routine histopathology is
not sufficient or the material for the analysis is very small (4).

Laryngeal carcinoma is one of the most frequent carcinomas of the head and neck. At present, laryngeal carcinoma is the fourth most frequently occurring carcinoma in males, with a clearly increasing occurrence. The assessment of gene expression profile shall allow to implement molecular biology methods in diagnostics, as well as in prediction of the course of disease. Thus, it may facilitate the most optimal decisions in regard to the method of treatment, extent of surgical procedure, or the necessity of adding post-operative radiotherapy (5, 6). The aim of the project was to analyse the gene expression profile in laryngeal carcinoma using oligonucleotide microarrays to derive novel molecular markers for that carcinoma.

MATERIAL AND METHODS.

The study group comprised of 14 patients (12 males and 2 females) with squamous cell laryngeal carcinoma, diagnosed and surgically treated between 2005 – 2007 in the ENT Department of the Silesian Medical University in Katowice, Poland.

Histopathology assessments were carried out in a typical manner: specimens were fixed in 10% buffered formalin and embedded in paraffin blocks, cut into ultra-thin paraffin sections of 3–4 micrometers and stained with hematoxylin and eosin (HE).

Tissue fragments (tumour and corresponding surrounding tissue) for molecular examinations were, immediately after collection, stored in liquid nitrogen. Total RNA was isolated both from tumour sections and corresponding fragments of macroscopically unchanged laryngeal tissue, collected from the same patient. RNA isolation was performed from about 40 mg of frozen tissue, by RNeasy Midi and Mini Kit (Qiagen) columns. For the synthesis of DNA (cDNA) 5 ug of RNA was used (GeneChip® Expression 3’ Amplification One-Cycle cDNA Synthesis Kit, Affymetrix). Then, in vitro transcription was carried out, in order to obtain biotinylated cRNA (GeneChip® Expression 3’ IVT Labeling Kit, Affymetrix). The amplified cRNA underwent fragmentation in high temperature/ Mg2+, which was followed by a 16–hour hybridization with high density microarrays Human Genome U 133 Plus 2.0 (Affymetrix) in the temperature of 45ºC. Arrays were stained by streptavidin-phycocerythrin complex. Scanning was carried out on GeneChip Scanner 3000 (Affymetrix). The quantity and quality of obtained RNA and cRNA were assessed using the Bioanalyser Agilent 2100.

14 tumour and 14 corresponding adjacent, apparently healthy tissue fragments after confirmation of RNA quality were hybridized to microarrays. The RNA quality index (RIN, ranging from 1 to 10) in most cases was within the range of 6.7–8.5, confirming the good quality of RNA, only for two normal samples it was below 6. Additionally, reference commercial RNA isolated from normal larynx was analyzed (Stratagene). In total, hybridization has been performed in 29 RNA samples. The prepared microarrays have been subjected to stringent quality control using the software affyQCReport. All microarrays complied with quality criteria, allowing to perform analysis of obtained data.

Microarray data were pre-processed by RMA algorithm, using Bioconductor 1.5 packages. The expression data were transferred to GeneSpring 6.2 and BRB Array Tools (developed by Dr. Richard Simon and BRB Array Development Team), where the analyses were carried out. Statistically significant genes were selected by the supervised analysis using Mann-Whitney non-parametric test and Welch t-test with Benjamini-Hochberg multiple comparisons correction, with the criterion of false positives percentage FDR<10%. Gene selection was also performed by Bayesian method with moderated t-statistic and FDR estimation (limma, linear models for microarrays). Class prediction analysis was carried out with Support Vectors Machines method. On selected genes we carried out the analysis of gene ontology.

RESULTS

In first step, we analyzed the obtained microarray data using unsupervised approach by Principal Component Analysis (PCA). At the interpretation of the 2-D Principal Components plot (see Fig. 1) we noted that the major source of variability in the analyzed dataset is not related to tumour-normal difference (samples in Principal Component #1 axis do not subdivide according to their origin). However, the difference between laryngeal cancer and unchanged adjacent laryngeal tissue is clearly visible in Principal Component #2, although there are some outlier samples. Three of the outliers are cancerous samples clustering with normal tissues, while one is normal specimen appearing between tumours. Two normal samples are half the way between the two populations. Four outlier samples (denoted by the arrows on Fig. 1) were excluded from further supervised analysis. To fully confirm the validity of our experiment, we obtained normal laryngeal tissue from commercial
source (collected from a person not suffering from laryngeal carcinoma, Stratagene) – this sample was co-clustering with our normal samples, although it was very close to one of the outlier tumour samples. In all the outlier specimens cancer was confirmed by histopathological examination, although it could not be excluded that sections used to microarray analysis contained the small admixture of either normal or cancerous tissue.

It has been found that, besides the differentiation observed between samples of neoplastic tissue and normal larynx, also another differentiation pattern has been observed, visible in the first component of PCA, dividing both the carcinoma group and, even more clearly, the group of non-transformed tissues into two subgroups. We could not identify the reason for this differentiation.

By analysis of the pattern of principal components (Fig. 2), we noted that both first and second component carry on the information about the tumour/normal difference and it is possible to classify the non-outlier samples based on the unsupervised analysis.

The supervised analysis based on non-parametric Mann-Whitney test selected 1241 transcripts, differentiating between both groups, at FDR<5%. 398 genes differentiating between laryngeal carcinoma tissues and tissues collected from the cancer surroundings fulfilled also the criteria of FDR<5% in parametric Welch t-test. In this analysis, a similar percentage of genes with increased and reduced expression in carcinoma was seen (Fig. 3).

For the final selection of genes, we used very simple criterion of non-corrected p value <0.001 in parametric t-test with random variance model. In this analysis we expect to obtain no more that 57 false positive transcripts; in fact we are obtaining 3241 probesets, confirming the large scale of transcriptome differences between laryngeal cancer and non-cancerous laryngeal mucosa. We performed hierarchical clustering of samples based on gene expression values of first 600 probesets from this analysis (Fig. 4). We obtained two very distinct gene expression clusters, although, we noted that two normal samples (not observed as outliers in unsupervised analysis) co-cluster with tumour samples, but exhibit a weaker intensity of cancer-specific gene expression signature.

We evaluated class prediction ability of examined specimens by Support Vector Machines algorithm, as implemented in GeneSpring 6.2. We have found that a majority of samples were classified correctly, only 2 of the 25 laryngeal samples from tumour surrounding were classified as tumour samples, similarly to the results seen in hierarchical clustering (data not shown).

To evaluate which biological processes are highly represented within the selected genes we used gene ontology over-representation analysis. Ontology classes significant for genes differentiating between laryngeal carcinoma and adjacent tissue are given in Table 2. The most significantly over-represented ontology classes are related to cell growth and its regulation and cellular communication.

8 genes selected by supervised analysis were chosen to be validated by real time RT-PCR (see Table 2). In four of them we confirmed the significant differences by independent method of measurement: those are: metal-proteinase ADAM12, cycline-dependent kinase 2 - CDK2, kinesine 14-KIF14, suppressor 1 of checkpoint - CHEK1. For the remaining genes (ATP6V1C1, RERG, ISG20L, and C18orf21) we failed to confirm by Q-PCR the differences in their expression between cancerous and adjacent laryngeal tissue.

**DISCUSSION**

Tumours classified clinically into the same category based on histopathologic characteristic may have different course of disease and respond differently to treatment applied. The introduction of oligonucleotide microarrays allowed identification of tumour subclasses within different malignancies, which substantially differ from the traditional
classifications. This lead to significant change of clinical practice, for example in breast cancer. Initial trials with DNA microarrays proved useful in subdividing leukemias, lymphomas or sarcomas. Correct diagnosis of a tumour type can be made following an analysis of expression of numerous genes. The use of the method will allow tumour differentiation and identification of new tumour subtypes.

Quantitative and qualitative aspects of microarrays can be used in screening tests in an attempt to identify molecular markers of head and neck squamous cell carcinoma (HNSCC). A strongly expressed transcript, specific for all HNSCC-involved tissues, but absent in healthy tissues, would be an ideal candidate to aid the diagnostic process. Molecular tumour profiling would make it possible to establish accurate diagnosis; it could also provide detailed information facilitating the development of a therapy targeted at molecular abnormalities.

Gene overexpression in laryngeal carcinoma was a subject of numerous research studies. Sok et al. (7) discussed two genes with a reproducible pattern of expression enhancement in laryngeal carcinoma while practically undetectable in healthy epithelium. The first was a human collagen gene, pro-alpha 1 (XI) (COL11A1), encoding an essential component of interstitial extracellular matrix. The other was a cDNA clone identified in human myeloblasts (KG–1). As it is probably a newly discovered gene, the authors refer to it as a HNSCCA – 1.

Fig. 2. Principal Components Analysis – pattern of first 6 principal components. PC1 and PC2 both carry the information about the difference between neoplastic and non-neoplastic tissues.

Fig. 3. Patterns of gene expression for all genes from supervised analysis. Outlier samples clearly visible (notice samples marked by no. 3 and 5, within normals)
Villaret et al. (8) identified 13 genes overexpressed in HNSCC when compared to control tissues. Nine of the genes were previously attributed to gene expression in HNSCC, ie. K6/K16 keratin markers, laminin-5, plakophilin-1, matrix metalloproteinase-2, vascular endothelial growth factor (VEGF), connexin 26, 14-3-3 sigma (p53), and CaN19. The remaining four are new.

Using cDNA microarrays containing 12530 genes, Al Moustafa et al. (9) compared HNSCC-involved and healthy tissues of the same patient. They demonstrated significant changes in the expression of 213 genes; 91 had increased, and 122 decreased expression. Two noticeable genes of decreased expression in HNSCC are Claudin-7 and Connexin 31.1. Sok et al. (7) also observed a decrease in Claudin-7 expression.

Belbin et al. (10) compared global gene expression patterns in tumour and healthy tissue samples, and distinguished two distinct genotypic subtypes of HNSCC. Although group I patients were younger and had poorly differentiated tumours, the group generally included early stage tumours and fewer lymph node metastases at presentation. The researchers isolated representative subsets of genes which divided the study group into two groups. Group I-specific genes were carcinoembryonic antigen (CEA), a member of the steroid receptor coactivator-1 family (AIB1), an amino acid transporter SLC7A8, ribosomal proteins S6 and S19, cytokine subfamily members such as SCYA16, PDE genes (phosphodiesterases), and the product of the ephrin B1 gene. The authors concluded that patient division based on gene expression profiles was a better outcome predictor than clinicopathological criteria.

Cromer et al. (11) identified a gene expression pattern which discriminates between tumour and healthy tissue of the laryngopharynx. They selected 6
new genes that are overexpressed in laryngopharynx tumours, ie., EIF4G1, DVL3, EPHB4, MCM&,
BRMS1 and SART1, and could serve as potential
markers of squamous cell carcinoma. They also
compared patients with low-aggressive and
aggressive tumours (metastases at three years
following surgery were used as the differentiating
criterium), and identified genes whose expression
was a potential initiator of metastasis. These were
autotaxin (ATX), fusin (CXCR4), and IL8.

Leethanakul et al. (12) used cDNA microarrays
to compare gene expression patterns in affected and
healthy tissues of patients with head and neck
squamous cell carcinoma. They found 59 genes with
differential expression including cytokeratines 7, 13,
14, 15, 17, 18 and 19, cyclin D-1, β-catenin,
transforming growth factor β, VEGF, FGF, and Wnt.
Their results are in accordance with those of Al-
Moustafa et al. (9).

In our study, among the genes revealing highest
variability of differences is the gene HIF-1A, induced
during cell response to the condition of hypoxia, as
well as genes characteristic for extra-cellular matrix,
such as MMP 1 or MMP 12. In physiological
condition they play an important role in tissue
formation during embryogenesis, whereas in
pathological processes they may influence the process
of metastasis. The polymorphism of MMP 1 gene is
not clearly specified previously as associated with
HNSCC. Based on our results, we selected potential
new marker genes, and among them genes whose
expression is either increased in laryngeal squamous
cell carcinoma: metalloproteinase (ADAM12),
cyclin-dependent kinase 2 (CDK2), kinesin 14 (KIF14), or demonstrates a decrease: checkpoint
suppressor 1 (CHES1).

ADAM12 expression in laryngeal carcinoma was
strongly up-regulated. The main functions of the
protein include proteolysis and interactions with
integrins and syndecans, the processes which
regulate cell adhesion. Enhanced expression of the
metalloproteinase weakens cell-cell interaction
mechanisms and increases tumour aggressiveness
through augmentation of tumour growth, shortens
the in situ phase and accelerates the metastatic
process. Thus, tumour cell dissemination and
histological malignancy increase. ADAM12 over-
expression results in stromal apoptosis enhancement,
which, together with reduced apoptotic potential of
cancer cells, favours tumour progression cascade.

CDK2 expression in laryngeal carcinoma was
also enhanced. Together with cyclin E, this protein
causes the progression from G1 to S phase of the cell
cycle. Cyclins and cyclin-dependent kinases
overexpression initiated by mitogenic signals
stimulates uncontrolled proliferation of tumour cells.
CDK2 overexpression has been demonstrated to
significantly correlate with tumour size, location,
stage and biological malignancy, ie., metastases
to lymph nodes and distant organs.
The mitotic kinesin KIF14 gene, also overexpressed in laryngeal squamous cell carcinoma, is a marker of high-proliferation rate tumours; its overexpression may cause genetic instability, disturbances of cytokinesis, and the rise of aneuploidal cell populations.

CHES1 protein (also known as FOXN), participates in G2/M checkpoint regulation. DNA damage mediates cell cycle arrest. As expected, our own investigations revealed a decrease in CHES1 gene expression resulting in lower sensitivity to DNA damage. Thus, cell cycle and cell division continued.

No differences in expression of the remaining genes investigated were found in squamous cell carcinoma and healthy laryngeal tissues: subunit C1 of vacuolar proton-ATPase V1 domain (ATP6V1C1), chromosome 18 open reading frame 21 (C18orf21), interferon stimulated exonuclease gene 20-like 2 (ISG20L2) and RAS-like, estrogen-regulated, growth-inhibitor (RERG). This underlies the importance of validation of results from microarray studies before their incorporation into further testing in preclinical and clinical setting.

CONCLUSIONS

The analysis of gene expression profile in laryngeal carcinoma is an efficient tool to delineate novel molecular markers in head and neck cancer.

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REFERENCES


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