PhD Thesis

Investigation of Laryngeal and Hypopharyngeal Carcinomas by Immunohistochemical Methods and Comparative Genomic Hybridisation

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1. INTRODUCTION

The head and neck squamous cell carcinomas (HNSCCs) -accounting for approximately 500.000 new cancer cases annually- represent 2-3% of all human malignancies.

These tumors are characterised by frequent recurrence and secondary malignant tumor formation. The overall 5-year survival rate of HNSCC patients is one of the lowest among the major cancer types and has not improved during the last decade despite advances in the diagnostics and management of the disease.

The prognostic divergence of laryngeal and hypopharyngeal carcinomas is well known: hypopharyngeal tumors are characterised by frequent metastasis formation and local recurrence which is the source of the unfavourable prognosis of that HNSCC subtype. The overall 5-year survival rate of patients with laryngeal SCC is 40-50% which represents a relatively good prognosis. However in case of hypopharyngeal SCC the overall 5-year survival rate is significantly lower: 10-15%.

In Hungary the occurrence of HNSCCs has increased during the last decades. During the past 25 years incidence of laryngeal carcinoma increased 2.5 times whereas incidence of hypopharyngeal tumors increased 5 times. 10 % of the death rate caused by cancer is formed by laryngeal and hypopharyngeal SCCs.

The molecular characterisation of laryngeal and hypopharyngeal carcinomas -similarly to other tumor types- would be desirable during diagnostic and therapeutic procedures. The most important would be to define the potential risk of local metastasis formation as the prognostic factors generally accepted for these tumors are the existence or lack of regional metastasis and localisation of the primary tumor.

Progression of malignant neoplasms is accompanied by alteration of the extracellular matrix (ECM) composition. The tumor stroma consists of normal extracellular matrix components as well as newly expressed components (ie. fibrin, tenascin) during tumorigenesis. The tumor matrix plays crucial role during inter-cellular communication, cellular differentiation and tumor cell proliferation.

Tenascin (TN) is known as a member of the adhesion modulating family of ECM macromolecules, thus its expression and distribution may have significant influence on tumor cell proliferation and invasiveness.

In normal adult tissues TN can be detected only in trace amount, but it is abundantly present in different embryonic tissues, especially in areas of epithelial-mesenchymal interactions, and in association with cell migration pathways. Recently a significant amount of data has accumulated in the literature, showing that the TN gene can be reexpressed or even upregulated in adults with certain pathological conditions characterised by cell proliferation, migration and extracellular matrix remodelling, such as wound healing and tumor growth. TN has several cell-binding domains that mediate cell adhesion, migration, and spreading, as well as matrix molecule (fibronectin, proteoglycan) binding regions by which it can interfere with the cell-binding function of fibronectin. It is worth mentioning that TN itself -possibly via EGF-like repeats- can directly stimulate the proliferation of certain tumor cells. Thus enhanced expression of TN is considered to be unfavourable prognostic indicator in malignancies.

Cancer occurs when cells escape the controls regulating the cell cycle and are able to proliferate regardless of signals from their environment. It is apparent from a variety of evidence that the development of cancer is a multistep process. Clinical neoplasms are comprised of a population of identical cancer cells known as a clone derived from a common ancestor. The initial genetic change in cancer transformation is thought to provide a growth advantage for the altered cell and its progeny. Although each new generation of daughter cells retains the original mutation, the opportunity exists for individual cells in the clone to sustain additional alterations that provide further growth enhancement. This results in the successive overgrowth of increasingly altered subclones that soon outnumber the less abnormal parent population, a phenomenon known as clonal expansion. The genes involved in cancer generally encode proteins involved in the key cellular pathways such as those that regulate the cell cycle, cell growth, or differentiation. Proto-oncogenes are normal cellular genes that influence cell growth in a positive way. When these genes are altered, becoming oncogenes, the corresponding protein function is increased. Tumor suppressor genes encode proteins that

normally exert a negative regulatory control on the cell. When these genes are altered, the control on growth is lost. A better understanding of events that take place during neoplastic transformation, invasion, and metastasis is needed to develop novel approaches to cancer diagnosis and treatment.

Initially standard cytogenetic analysis was applied to the investigation of HNSCCs. Analysis showed that cell populations with malignant behaviour of HNSCCs are generally hyperdiploid to near tetraploid, carrying a large number of structural abnormalities on different chromosomes. Although these investigations have been a significant step in the identification of genetic changes of HNSCCs, the difficulties in culturing and the presence of polyclonal cell populations limit the usefulness of these results. It was also shown that manipulations in culturing conditions can alter the karyotypic findings in solid tumors, including head and neck carcinomas.

In order to define further chromosomal alterations a number of recently developed molecular genetic techniques were applied like FISH and CGH.

It is well known that comparative genomic hybridisation is one of the most useful technique to identify chromosome regions affected by malignant transformation. It enables the total mapping of chromosomal copy numbers in the entire tumour genome and it is not restricted to *in vitro* artificial conditions.

Using CGH technique it was shown that a relatively large number of chromosomal regions are consistently altered in HNSCCs.

Although head and neck tumors can be located in many different sites, the genetic alterations of the different tumor subgroups based on localisation were separately analysed only in a few CGH study, and the authors could not clarify characteristic differences between the investigated tumor types.

Although the prognostic divergence of carcinomas originating from the larynx and hypopharynx is well-characterised, our knowledge about the genetic alterations of these two groups are insufficient. Therefore the aim of this investigation was to compare the chromosomal alterations mapped by CGH of these two subgroups and to find new chromosomal loci that might be characteristic for the aggressive phenotype. This is the first CGH investigation in which CGH data of these two HNSCC subgroups are compared. We also analysed the relationship of the laryngeal and hypopharyngeal carcinomas to clinical parameters to improve prognostic assessment in order to find clinically

relevant genetic alterations that might be responsible for the development and aggressive behaviour of these neoplasms.

2. AIMS

Our aims were:

- to define the distribution pattern of TN in laryngeal and hypopharyngeal cancer samples. In double and triple immunofluorescent staining reactions the detection of TN was combined with labellings for cytokeratin, CD-34 and reaction with Ki-67.
- to determine TN distribution in comparison with tumor stage and metastasis formation.
- to elucidate the prognostic significance of TN production.
- to define characteristic genomial alterations in laryngeal and hypopharyngeal cancers with the help of CGH.
- to determine characteristic genomial alterations that could be responsible for the more aggressive behaviour of hypopharyngeal tumor cells.

3. MATERIALS AND METHODS

Tissue Specimens

Samples of head and neck squamous cell carcinomas (n=58; laryngeal n=31 and hypopharyngeal n=27) were obtained from the Department of Otorhinolaryngology Head and Neck Surgery, University of Debrecen, Hungary with the approval of the Committee on Human Experimentation of the Medical and Health Science Center, University of Debrecen.

The clinical records of all patients were available in the same Department. Patients had not been given chemotherapy or radiotherapy before surgery. Clinical staging was determined by TNM classification of cancer. Tumor samples were classified on the basis of locations, TNM stages and data concerning grading. Histological diagnosis was made on preoperative biopsy specimens by the Department of Pathology.

Tumor tissue samples were immediately frozen after the surgery and stored at -86°C. 5-7 micron sections were stained with haematoxylin and eosin for morphological examination. Histopathological classification was performed according to the WHO International Classification of Tumors.
Non-specific immunoglobulin-G binding was blocked by preincubation with 5% normal human serum diluted in iso-osmolar phosphate buffered saline, pH 7.4.

Double Immunofluorescent Labelling

Double immunofluorescent staining reactions were carried out to detect simultaneously:

-TN and cytokeratin (marker protein of epithelial cells).

-TN and Ki-67 monoclonal antibody (nuclear antigen in proliferating cells).

Sections were incubated with a 1:200 dilution of mouse anti-human TN and in the second reaction rabbit anti-human pan-cytokeratin in a 1:100 dilution. The reaction for TN was visualised by Texas red streptavidin (1:40). To detect the binding of cytokeratin antibodies, anti-rabbit IgG conjugated with FITC was used in a 1:40 dilution.

For simultaneous detection of cytokeratin and the nuclear antigen of proliferating cells, the above mentioned reaction was combined with Ki-67 reaction, in which 1:100 dilution of Ki-67 monoclonal antibody was used, and its specific binding was detected by the indicator reactions described above for TN.

Triple Immunofluorescent Labelling

Triple immunofluorescent staining reactions were carried out to detect simultaneously:

-TN, cytokeratin and CD-34 (endothelial cell surface glycoprotein).

-TN, CD-34 and Ki-67 monoclonal antibody (nuclear antigen in proliferating cells).

For TN, cytokeratin and CD-34 sections were incubated with a 1:200 dilution of mouse anti-human TN and rabbit anti-human pan-cytokeratin in a 1:100 dilution. The reaction for TN was visualised by biotinylated anti-mouse IgG (1:250) and AMCA-avidin (1:80) while anti-rabbit IgG conjugated with FITC (1:40) was applied. Than 1:2 dilution of anti-CD-34 conjugated phycoerythrin was used.

The triple immunofluorescent staining for TN, CD-34 and Ki-67 was carried out similarly, to the previous reaction, differing only by substituting 1:100 dilution of Ki-67 monoclonal antibody for the TN reagent.

Immunohistochemical Analysis and Documentation

After stainings sections were covered with PBS-glycerol and evaluated in an Axiooplan fluorescence microscope equipped with an epifluorescence condenser containing selective filters. Images were captured by using ISIS 3 software, digitised and stored on floppy disks.

Comparative Analysis of Clinical and Immunohistochemical Data

Clinical and immunohistochemical data were combined and TNM stage, recurrence and death rate were determined in each category defined on the basis of TN distribution pattern for both laryngeal and hypopharyngeal cancers. For statistical analysis, the F-probe test was used.

Tumor Samples investigated by CGH

Samples of head and neck squamous cell carcinomas (n=23; laryngeal n=14 and hypopharyngeal n=9) were obtained from the Department of Otorhinolaryngology Head and Neck Surgery, University of Debrecen, Hungary and Department of Otorhinolaryngology Head and Neck Surgery, Semmelweis University, Hungary with the approval of the Committee on Human Experimentation of the medical and Health Science Center, University of Debrecen.

The clinical records of all patients were available in the same Departments. Patients had not been given chemotherapy or radiotherapy before surgery. Clinical staging was determined by TNM classification of cancer. Tumor samples were classified on the basis of locations, TNM stages and data concerning grading. Histological diagnosis was made on preoperative biopsy specimens by the Department of Pathology.

Tumor tissue samples were immediately frozen after the surgery and stored at -80°C until the CGH experiment. Six micron sections were stained with haematoxylin and eosin for morphological examination. Histopathological classification was performed according to the WHO International Classification of Tumors.

Preparation and Labelling of DNA.

In all cases of tumors test high molecular weight DNA was extracted from 5-10 pieces of 30 micron frozen tissue sections of tumors. The concentration of DNA was determined in a fluorimeter. Reference DNA was extracted from peripheral blood of a healthy volunteer according to a standard procedure.

Tumor DNA was directly labelled with SpectrumGreen-12-dUTP and normal DNA was labelled with SpectrumRed-5-dUTP by nick translation. The experimental conditions were adjusted to gain DNA fragments of 600-2,000 bp.

Comparative Genomic Hybridisation.

The hybridisation mixture, consisting of 200 ng of each labelled DNA and 20 μg of unlabelled human Cot-1 DNA were precipitated and dissolved in

10 μl of hybridisation mixture and denatured at 73°C (3-5 min) immediately before being applied onto normal metaphase spreads. Slides with target metaphase chromosomes, were denatured at 73°C for 3-5 min, dehydrated through ethanol series, and air-dried. Hybridisation was carried out at 37°C in moist chamber for 72 h. Then slides were washed three times in hybridisation wash buffer, twice in PN buffer at RT for 10 min each, and then air-dried. Nuclei were stained with 0.15 μg/ml 4,6-diamino-2-phenylindole in anti-fade solution. One negative (differentially labelled, normal DNA vs. normal DNA) and a positive control (SpectrumGreen-12-dUTP labelled, cytogenically well-characterised breast cancer cell line: MPE-600) were included to monitor hybridisation quality. Only images showing uniform high-intensity fluorescence were analysed.

Digital Image Analysis and Interpretation of CGH Data.

A multicolour quantitative image processing system (ISIS) connected to a Zeiss fluorescence microscope was used for acquisition and for evaluation of the CGH experiment. Gray level images of the three colour (DNA counterstain blue and the fluorochromes SpectrumGreen for tumor DNA and SpectrumRed for reference DNA) were acquired from at least 10 metaphases using a high-sensitivity monochrome charge-coupled-device. After automatic interchromosomal background subtraction, chromosome segmentation was carried out based on the DAPI image. Chromosomes were identified on the basis of the DAPI image. Fluorescence intensity profiles of green and red fluorescence were calculated and the green-to-red ratios was plotted. The lower and upper threshold values were defined as described. Gain of chromosomal regions was assumed at ratio of >1.15 and loss was defined at ratio<0.85. Amplifications were assumed only when the green to red ratio was above 1.5 or visual inspection revealed a bright and distinct green fluorescent signal at a subregion of the homologue chromosome. Chromosomes and copy number changes at the heterochromatic region or the p-arms of acrocentric chromosomes were excluded from the analysis because these regions were effectively blocked with Cot-1 DNA resulting in very low signal intensities.

Statistical Analysis.

Chromosomal gains and losses of the two HNSCC subgroups were compared by F-test, P values less than 0.05 were considered significant. Chromosome copy number changes with tumor locations, metastasising and hystological type of the disease were correlated. As follow up period was not sufficient, time of progression and survival has not been analysed.

4. RESULTS

Characteristic Distribution Patterns of TN in Laryngeal and Hypopharyngeal Cancers

The mean ages were 55 years for patients with laryngeal carcinoma and 50 years for patients with hypopharyngeal tumors. The difference was not significant statistically.

By immunohistological methods in laryngeal cancers, in early stages of tumor growth a markedly enhanced production of TN at the tumor host interphase was observed. In the later stages of tumor progression a high number of blood vessels located in the tumorous tissues were also strongly labelled for TN. Around these vessels a significant number of proliferating tumor cells could be detected. In contrast, in hypopharyngeal cancers this vasculature-associated staining pattern could be observed from the very early stage of tumor development. In laryngeal and in hypopharyngeal cancers TN upregulation strongly correlated with metastasis formation, early tumor recurrence and lethal outcome of the disease.

Genetic Alterations Detected in Laryngeal Carcinomas by CGH.

Fourteen laryngeal carcinoma were analysed by CGH. All tumors showed chromosome copy number alterations by CGH. The average number of genetic alterations/tumor in laryngeal carcinomas was 5.36 (range 1-10). Overrepresentation of DNA sequences varied between 1 to 7, with an average number/tumor of 3.07. Losses were seen relatively less frequently, 2.14 losses (range 0-7) per tumour were detected. Only a few chromosomes did not exhibit any alterations including chromosome 6, 15, 19, 20 and 21.

Chromosomal copy number alterations were detected on chromosome 3q and 8q with a same frequency (57%, respectively). The gain of the long arm of chromosome 3 was associated with the complete deletion of the short arm of the same chromosome in 2 cases (14%). Similar alteration was seen only once on chromosome 8 (7%). The second most frequent alteration was the gain of 11q (50%). The over-represented common region of this chromosome region involved the 11q13 segment in six tumors (43 %), this amplified part was well visible, appearing as bright green fluorescent band

under the microscope. Two-third of 11q13 gain was associated with the deletion of the distal end of chromosome 11q. In three tumors (21%) the gain of chromosome 5p, 9p and in two carcinomas (14%) the overrepresentation of 2q and 7p were also observed. The gains of other chromosomal regions involving 1q, 7q, 12p, 12q, 13q, 14q, 17p, 17q, 18p, 18q were seen only in one case, respectively.

Under-representation of the short arm of chromosome 3 and the long arm of chromosome 11 were the most frequent alterations, 43% of larynx tumors exhibited copy number changes on these DNA segments. The minimally deleted region on chromosome 11q was mapped to 11q23-11qter. Loss on 8p was seen in 3 cases (21%) and losses on 5q, 9p and 18q were detected in 2-2 tumors (14%). Other losses were found on 2q, 4p, 7q, 10q, 12q, 16p, 16q, 17p, 22q only in one case, respectively.

Genetic Alterations in Hypopharyngeal Carcinomas Revealed by CGH.

A total of 9 primary hypopharyngeal carcinomas were investigated for relative DNA alterations by CGH. All tumors showed chromosome copy number alterations on two or more DNA region. The average number of genetic alterations per tumor was 5.75 (range 1-11). The number of gains and losses of DNA sequences were similar. On average, 3.37 gains (range 1-6) and 3.0 losses (range 1-5) per tumor were found. With the exception of chromosomes 13, 14, 15, 19, 20, 21 and 22 all chromosomes were affected by alterations.

The most frequent chromosomal copy number change was detected on the long arm of chromosome 3 with a frequency of 77%. The entire long arm was over-represented only in 2 tumours, the commonly gained DNA segment was mapped to 3q21-qter. Chromosome 3q gain was associated with loss of chromosome 3p in 6 tumours (67%). Gain of the long arm of chromosome 3 was associated with the complete deletion of the short arm in two cases. The second most frequent alteration was the overrepresentation of 11q in 3 cases (33%), involving the 11q13 region. The gain of 11q13 was associated with the deletion of the distal end of chromosome in one case (11%). Over-representation on 7q and 12p could be observed in two tumors (22%). The

gain on 1q, 2p, 2q, 4q, 5p, 6q, 7p, 8p, 9q, 12q, 16q, 17q and 18p were seen only in one case, respectively.

Loss of the short arm of chromosome 3 was the most frequent alteration with a frequency of 66%. Losses on 9p and 18q occurred in 3 cases (33%) and loss on 1p, 2q, 4p and 7q in 2-2 cases (14%). Under-representation of 5q, 10q, 11q, 12q and 17p were detected only in one case, respectively.

Comparison of Genetic Alterations of Laryngeal and Hypopharyngeal Tumours.

The average number of genetic alterations were not significantly different between laryngeal and hypopharyngeal tumors (P=0.094). Similarly to this no significant difference were observed between the two subgroups for chromosomal gains and losses (gains: P=0.068, losses: P=0.49). Chromosome copy number gains of 3q was the most frequent alteration in both tumour type but in hypopharyngeal cancers this alteration was more frequent (77%) compared to laryngeal tumours (57%). Gains of the chromosome 11q region was present in half of the laryngeal samples (50%) and only one-third (33%) of the hypopharyngeal tumors exhibited aberration in this region. Chromosome alteration that was present with a relatively high frequency (21%) in the laryngeal subtype but was absent from the hypopharyngeal subgroup was the loss of chromosome 8p. Loss of the distal part of the long arm of chromosome 11 (11q14-qter) was more characteristic for the laryngeal tumours, 43% of tumors have lost this part of the genome whereas only 11% of hypopharyngeal tumors exhibited this alteration. Chromosomal copy number changes that were not detected in hypopharyngeal tumours but was seen in more than 50% of laryngeal cancers was the gain of the long arm of chromosome 8.

5. DISCUSSION

Our results proved the unfavourable prognosis of hypopharyngeal cancers compared with laryngeal ones.

By immunohistological methods in laryngeal and hypopharyngeal cancers we observed enhanced TN production in correlation with tumor progression. We showed close relationship between TN production and angiogenesis, as well as metastasis formation. Clinical and immunohistologic data indicate that the accumulation of TN in the tumor blood vessels is an unfavourable prognostic indicator in laryngeal and hypopharyngeal cancers.

By CGH analysis of the tumors we found that the average number of genetic alterations did not differed significantly in the two subgroups. However, clear difference was found by the analysis of the distinct chromosomes. Overrepresentation on 3q was the most frequent alteration in both tumor type but this alteration was more frequent in hypopharyngeal cancers. Gains of 11q13 with or without the loss of the distal end of chromosome was more characteristic for laryngeal tumors. This type of alteration is thought to be related to enhanced metastasis formation but our results did not supported this hypothesis. Chromosomal alteration which was not detected in hypopharyngeal tumors but was seen in more than 50% of laryngeal carcinomas was the gain on 8q. This chromosome region harbours *C-MYC* but the function of this oncogene has not been clarified yet. In our samples overrepresentation of this region was accompanied by reduced metastasisation. In conclusion our data suggest that not only the prevalence but also the prognostic significance of chromosomal alterations varies between differently localised tumors of the upper aerodigestive tract.

List of publications

Publications related to the thesis:

Juhász A, Bárdos H, Répássy G, Ádány R (2000): Characteristic Distribution Patterns of Tenascin in Laryngeal and Hypopharyngeal Cancers. The Laryngoscope. 110:84-92.

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