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Gene Amplification of Epidermal Growth Factor Receptor in Atypical Glottic Hyperplasia

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A B S T R A C T

The study searched for epidermal growth factor receptor (EGFR) gene amplification in hyperplastic glottis lesions. After classical pathohistological findings of hematoxylin-eosin (HE) slides and quantitative immunohistochemical (IHC) analysis, fluorescent in situ hybridization (FISH) was used on tissue microarrays of laryngeal hyperplastic tissue ranging from normal mucosa to abnormal and atypical hyperplastic lesions. FISH analysis of two atypical hyperplastic lesions discovered the amplification of EGFR gene while it was not found in simple and abnormal hyperplastic lesions. The results may indicate that EGFR gene amplifications could possibly correlate with the histopathologic picture. Tissue samples burdened with specific oncogen signatures like EGFR gene amplification could be detected in precancerous lesion. This might improve follow-up and treatment protocols of glottic lesions which are an everyday problem for ENT practitioners. Further research is mandatory to confirm our findings.

Key words: *EGFR, FISH, gene amplification, hyperplastic glottic lesions, precancerous*

Introduction

Over-expression of EGFR has been found in precancerous laryngeal lesions and connected with their advancement and aggressiveness[1–3]. The relation between gene amplification and protein over-expression remains unclear.

Studying EGFR impact on cancerogenesis, scientists have shown that protein over-expression is not precise enough for the selection of patients for treatment protocols. Researches indicate that gene alterations can be useful for the selection of patients suitable for new anti-EGFR therapies[4–8].

There are only a few reports about gene amplification in laryngeal cancer[9–11], and it has not been researched on hyperplastic glottic tissue. Laryngeal cancer shows a lot of variability and often multifocal growth also. The bioptician must bear this in mind. It is known that different anatomic locations of laryngeal tumours (glottis, supraglottis, subglottis) have different biological behaviour (metastases, spreading to surrounding tissues). The glottic area is specific since it has the best prognosis. Precancerous lesions of the glottic region with hoarseness as an early symptom represent one of the major problems of every day ENT practice. It is important as it is the site of origin of over 60% of laryngeal cancers and because of its impact on patients' quality of life[12]. This is why we have investigated EGFR in glottic precancerous lesions.

To evaluate EGFR gene amplification in a large number of glottic hyperplastic lesions, we have used fluorescent *in situ* hybridisation (FISH) on tissue microarrays, and have compared the results with classical pathohistology and immunohistochemistry EGFR protein levels.

Patients and Methods

The research was done on biopsy samples of glottic hyperplastic tissue harvested from the Department of Pathology and Otorhinolaryngology of the Rijeka University Hospital Center with the Approval of the Ethics Committee.

The biopsies were obtained through direct laryngoscopy from a vast pool of clinical diagnoses of prolifera-

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tive vocal cord lesions ranging from polyps, oedemas, papillomas, Singer's nodules, chronic hypertrophic laryngitis, leukoplakias and suspected neoplasmas. Slides stained with hematoxylin-eosin were reviewed by an experienced pathologist who confirmed the histopathological diagnoses.

The hyperplastic lesions were graded according to the Gale-Kambič-Lenart Ljubljana classification[13] and subsequently divided into groups.

Analysed tissue samples which had simple hyperplasia as the highest mucosal change, were taken as control samples. Abnormal and atypical hyperplasias were analysed as precancerous lesions. From a total of 125 HE slides that entered the study, 41 control samples and 60 precancerous lesions (29 abnormal and 31 atypical hyperplasias) were selected by the pathologist for further IHC and FISH analysis. Seventy-three isolated tissue specimens of hyperplastic lesions, among which 41 simple, 15 abnormal, and 17 atypical hyperplasias were examined. Additionally, for 14 patients with developed laryngeal squamous cell carcinoma (SCC), adjacent areas of tissue presenting with different grades of hyperplastic lesions were analysed (14 abnormal and 14 atypical hyperplasias).

IHC analysis was successfully performed on all bioptic material, while there was some tissue loss during FISH procedures.

In the control group there were 41% biopsies taken from male patients and 59% biopsy samples from women. In precancerous lesions there were only 5.6% female patients, the rest were male, confirming the fact that glottic precancerous and cancerous lesions are predominant in the male population. The range was from 30 to 75 years of age in the control group and from 42 to 79 years of age in patients with precancerous lesions. There were 75% of smokers in the control group, and 77% of smokers with precancerous lesions.

Tissue microarray (TMA) construction

Haematoxylin-eosin (HE) stained sections of glottic mucosa were used to mark areas with surface epithelial layers, avoiding areas of necrosis. Three tissue cores, each 1 mm in diameter, were placed in a recipient paraffin block using a manual tissue arrayer (Alphelys, Plaisir, France). Normal liver tissue was used for slide orientation. Cores were spaced at intervals of 0.5 mm in the x- and y-axes. One section from each TMA block was stained with haematoxylin-eosin for morphological assessment. Serial sections were cut from TMA blocks for IHC staining. Five μm thick sections were placed on adhesive glass slides (Capillary Gap Microscope Slides, 75 μm, Code S2024, DakoCytomation, Glostrup, Denmark), left to dry overnight at 37°C and stored in the dark at + 4°C.

Immunohistochemical staining and evaluation of EGFR expression

Following deparaffinization in xylene and rehydration in alcohol, heat-induced epitope retrieval was achieved by immersing the slides in Tris-EDTA buffer (pH 9.0) and boiling them for 10 minutes in a water bath. Slides were allowed to cool over 45 minutes, and then pre-incubated for 30 minutes with blocking solution containing normal donkey (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat serum (DakoCytomation, Glostrup, Denmark), respectively. Sections were incubated with pre-diluted primary monoclonal antibodies for EGFR (EGFR pharmDx, Monoclonal Mouse antihuman EGFR (clone2-18C9)IgG_1). Antibody immunohistochemistry was performed in Dako Autostainer Plus (DakoCytomation Colorado Inc, Fort Collins, CO, USA) according to the manufacturer's protocol, using LSAB technology. All reagents for automated IHC were purchased from DakoCytomation (ChemMate TM Detection Kit K5001, ChemMate TM Envision HRP detection Kit K5007). Slides were counterstained with Mayer's haematoxylin, dehydrated and mounted. Negative control slides were prepared by substituting Dako ChemMate antibody diluent for a secondary antibody. Moreover, for positive and negative controls, colonic cancer tissue sections over-expressing EGFR and apparent normal colonic mucosa were used.

A quantitative analysis of EGFR expression was performed and membranous staining was evaluated. The cells were analyzed by layers. The basal zone was defined as cells with stromal contact; the superficial zone as surface cells with applanated nuclei, and the suprabasal zone was defined as the transforming layer between them. The results were analyzed on automated image analyzer using Issa software (VamsTec, Croatia). Statistical analysis was performed counting all cells on 0.01 mm^2 in defined zones of normal and hyperplastic tissue and calculating the percentages of positive cells.

Fluorescent in situ hybridization

After deparaffinization, the slides were pre-treated with Paraffin Pretreatment Kit (Vysis, Downers Grove, IL, USA) using protease to digest proteins for 10 min. FISH was performed by applying EGFR probe (Vysis, Downers Grove, IL USA): LSI EGFR SpectrumOrange and CEP 7 SpectrumGreen for respective centromeric region at chromosome 7. The LSI EGFR Dual Color probe hybridizes to the band region 7p12 in Spectrum Orange and the CEP 7 to the 7p11.1-q11, 1, D7Z1 locus in Spectrum Green. Denaturation of the probe mixture was performed at 95 °C for 5 min. in Thermobrite (Abbott Molecular Inc, IL, USA) followed by overnight hybridization at 37 °C. The following day, cover glass was removed and slides were washed in post-hybridization washing-buffer (Vysis, Downers Grove, IL, USA) at 72 °C for 2 minutes. After drying, DAPI (Vysis, Downers Grove, IL, USA) was applied and slides were protected from light at –20 °C until reading. Automated scanning, capture and scoring of interphase FISH was analyzed by Cytovision System (Applied Imaging Corp., San Jose, USA). EGFR gene amplification was defined as a ratio between EGFR gene (orange) and centromere (green)

signals in cell nuclei over 2.2, or the presence of EGFR gene clusters.

Statistical analysis

Statistical analysis between the three groups was carried out using Kruskal-Wallis test. For differences between single group pairs, the Mann-Whitney test was applied. The level of statistical significance was set at $P<0.050$. For statistical analysis, SPSS 15.0 software was used.

Results

IHC analysis was successfully performed on 41 simple, 29 abnormal and 31 atypical hyperplasias chosen by an experienced pathologist.

To evaluate membranous EGFR (mEGFR) protein over-expression through epithelial layers, we calculated the percentages of positive cells in the basal and suprabasal layers (2/3 of the epithelium) and all the layers (entire epithelial thickness), based on previous research[1–3] (Figure 1).

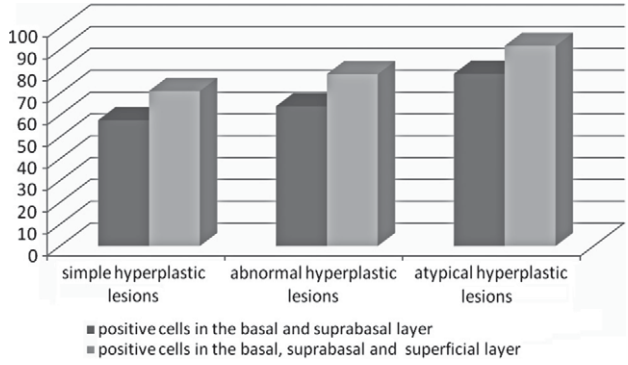

Fig. 1. Percentages of membranous positive cells for epidermal growth factor receptor (EGFR) in simple, abnormal and atypical hyperplastic lesions.

The percentage of positive cells in the basal and suprabasal layer (2/3 of the epithelium) and all the layers (entire epithelial thickness) was statistically higher in atypical than in abnormal hyperplastic lesions ($P<0.050$, Mann-Whitney test). Abnormal and atypical hyperplasias had statistically higher percentages of mEGFR positive cells in the basal and suprabasal layers as well as in the entire epithelial thickness than simple hyperplasias taken as control samples ($P<0.05$, Kruskal-Wallis test, Figure 1).

Membranous EGFR protein expression gradually increased from negative to weak positive and strong positive throughout all layers with increased atypia, indicating that mEGFR staining can facilitate the differentiation between different degrees of atypia among hyperplastic lesions.

No statistical difference of mEGFR protein expression was found between isolated hyperplastic lesions and specimens with the same degree of atypia adjacent to tumour samples.

Epidermal growth factor receptor gene in situ hybridization (FISH)

FISH was successful for 37 simple, 23 abnormal and 26 atypical hyperplastic glottic tissue samples. There was some loss of tissue material during TMA sectioning and hybridization procedures.

Amplification was detected in 2 tissue samples that showed 5 or more signals or clusters of EGFR gene.

There was no amplification in the control group and abnormal hyperplastic lesions (Figure 2a). Both amplifications were found in atypical hyperplasias (Figure 2b).

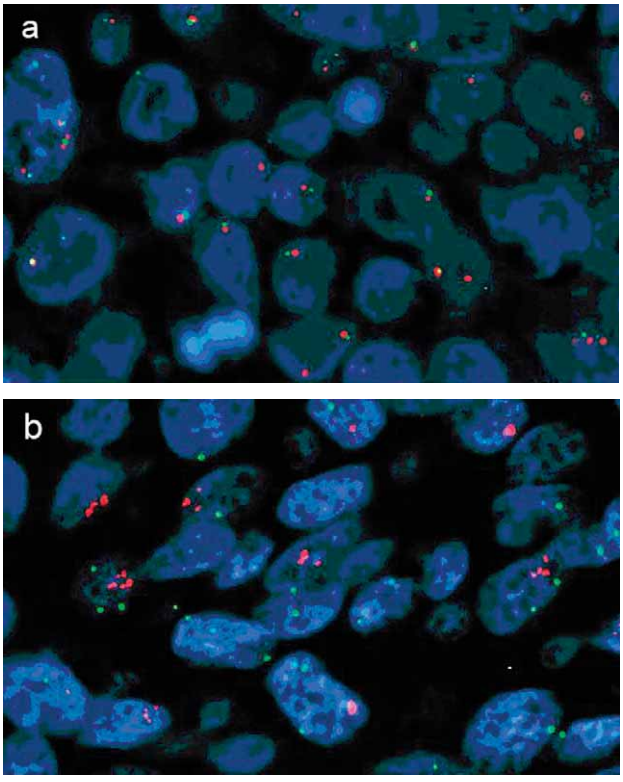

Fig. 2. Fluorescent in situ hybridization (FISH) for epidermal growth factor receptor (EGFR) gene in glottic lesions. DNA probes: Spectrum Orange-labeled for EGFR gene, and Spectrum Green-labeled centromeric probe for chromosome 7. a) Control sample: no EGFR gene amplification (magnification 100x). b) EGFR gene amplification in atypical hyperplasia: the score between EGFR gene (orange) and centromere (green) signals in cell nuclei over 2.2 (magnification 100x).

One entered the study under the clinical diagnosis of suspected neoplasma of the vocal cord. The other was found on atypical hyperplasia adjacent to SCC.

There was no statistical significance between mEGFR protein expression of amplified atypical lesions and those without EGFR gene amplification.

Discussion

In this study, we used tissue microarray technology (TMA) on samples of laryngeal mucosa ranging from simple, abnormal to atypical hyperplastic lesions.

Using FISH, it was possible to detect gene amplification in glottic lesions and compare results with standard pathohistology and IHC protein levels of EGFR.

Based on pathohistologycal findings, the distinction was made between abnormal and atypical hyperplasias because of their different biological behaviour. Abnormal hyperplasias can be regenerative, while atypical lesions have a more malignant potential and require different treatment. Hyperplastic lesions of the glottic region cover a wide spectrum of histomorphologic changes. In everyday practice, histopathology results based on traditional light microscopy represent the basis of diagnosis. For diagnostically difficult cases, nowadays pathohistology is not precise enough in protocol decisions, and IHC analysis is used in many clinical centres.

EGFR protein expression usually increases with degrees of atypia[1–3,14,15], as it was in this study. Our research did not find statistical differences in mEGFR protein levels of isolated laryngeal lesions with the same lesions adjacent to SCC as some authors' did[3]. The evaluation of EGFR protein over-expression can sometimes be difficult in a multilayered epithelium, and high levels of EGFR protein can often be found even in simple and abnormal hyperplastic lesions[1,3,14]. This research also detected high levels of EGFR protein in some control samples and abnormal hyperplastic lesions, but none of them showed gene amplification which seems to lead to the transformation of atypical lesions into cancerous tissue. It was detected in 2 highly atypical lesions. One of them developed into preinvasive cancer within one month of follow-up. The other was found in atypical hyperplasia adjacent to SCC. Although two cases of amplifications are a humble number, the fact that none of them was found in simple or abnormal hyperplastic lesions but only in the group of atypical lesions (that developed into or were found next to cancerous tissue) may indicate that amplifications could possibly correlate with the histopathological picture, making identification of biologically more aggressive lesions easier.

Detection of EGFR gene amplification might help the distinction of lesions that carry a higher risk of progression into cancerous tissue. This genetically changed and instable tissue could be diagnosed before its passage from malignant genotype into phenotype and help prevent cancer formation. Hypothesis of cancer development in the head and neck region have been discussed as early as the 1990. The field cancerisation concept suggests carcinogen exposed tissue accumulates genetic changes progressing from dysplasia (atypical hyperplasia) into cancerous tissue[16]. The second hypothesis presumes that head and neck cancer arise from micrometastatic foci of a single progenitor clone[17]. Research of a specific area such as the glottic region is lacking. Laryngeal cancer often shows multifocal growth. This must be taken into account when biopsies are taken. Often the pathologist can find different types of hyperplasias adjacent to cancerous tissue on the same slide. Cases of repeated relapses of atypical hyperplasias still divide clinicians on follow-up and treatment protocols. Perhaps the site of biopsy presents the hystologic picture of atypical hyperplasia but adjacent tissue that was not included in the specimen already holds the features of preinvasive tumour, and even though multiple and repeated biopsies are taken as a rule by most practitioners, mistakes in grading the aggressiveness of a lesion cannot be avoided in some cases. Researchers have previously investigated genetic instabilities in glottic lesions and chromosome 7 aberrations have been found in dysplastic and cancerous laryngeal tissue and connected with prognosis[3, 18,19], but gene analysis is insufficient. This is why we have investigated EGFR gene amplification in glottic lesions and EGFR gene amplification in atypical hyperplasias is an entirely new finding. Further research, which is currently being undertaken at our Clinic, is mandatory to confirm the significance of our findings.

Glottic precancerous lesions are important because of their high incidence and influence on quality of life. Selection of patients with atypical lesions that hold a more malignant potential would greatly help better follow-up and treatment of these lesions that represent a major problem in everyday ENT practice. In these lesions biomarkers like EGFR could help in decision making. Early glottic cancer (Tis-$T_1T_2N_0M_0$) is an entity that can be treated by radiotherapy or surgery. Literature data indicate that EGFR can help in the selection of patients suitable for radiotherapy[20,21,22]. The significance of EGFR gene amplification on laryngeal radiosensitivity has not been researched yet. Also, a new area of interest currently under investigation at our Clinic is nuclear EGFR protein expression, the role of which, according to published data, still remains unclear[23,24,25]. Our final purpose is to determine subpopulations of lesions with different EGFR status in order to optimize their treatment accordingly. This might be helpful for decision making in precancerous lesions, as well as defining therapeutic protocols for laryngeal cancer (radioresistance, involvement in monoclonal antibody treatments with regard to EGFR status).

The importance of glottic lesions for patient's quality of life raises the need for individualized therapies and optimal treatment protocols based upon molecular markers. We hope that our research can add to higher awareness of EGFR in glottic hyperplastic lesions. Still many open questions and insufficient data indicate the need for further research in this field.

Conclusion

For optimal treatment and follow-up of precancerous glottic tissue lesions, subpopulations of patients with specific molecular signatures are needed.

The study discovered two cases of EGFR gene amplification in atypical hyperplastic lesions. No EGFR gene amplification was found in simple or abnormal hyperplasias. Since IHC analysis of glottic hyperplasias sometimes lacks precision, our research indicates that in diagnostically difficult cases detection of EGFR gene amplification might point to the aggressive nature of the lesion, improving the accuracy of diagnosis and therapy choices of glottic lesions. Further research is needed to confirm the significance of our findings in glottic precancerous lesions

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GENSKA AMPLIFIKACIJA RECEPTORA EPIDERMALNOG FAKTORA RASTA U ATIPIČNIM HIPERPLAZIJAMA GLOTISA

SAŽETAK

Studija istražuje gensku amplifikaciju receptora epidermalnog faktora rasta (EGFR) u atipičnim hiperplazijama glotisa. Nakon klasične patohistološke i imunohistokemijske (IH) analize, korištena je fluorescentna *in situ* hibridizacija (FISH) na tkivnim mikroarejima hiperplastičnih glotičnih uzoraka različitog stupnja atipije. Genska amplifikacija nije nađena u jednostavnim i abnormalnim hiperplazijama.Otkrivena je u dvije visoko rizične atipične hiperlpastične lezije.Tkivni uzorci opterećeni određenim onkogenim promjenama poput EGFR genske amplifikacije mogli bi biti otkriveni u prekanceroznim lezijama, spriječivši njihovu malignu transformaciju. Navedeno bi moglo poboljšati protokole praćenja i liječenja glotičnih prekanceroznih lezija koje predstavljaju veliki problem svakodnevne ORL prakse. Daljnja su istraživanja potrebna da potvrde naše rezultate.