The Role of Nrf2 in Pathology of Pleomorphic Adenoma in Parotid Gland

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Background: Pleomorphic adenoma (benign mixed tumor) is one of the most common salivary gland tumors. However, the processes involved in its carcinogenesis are not well defined. This study aimed to define the contribution of Nfr2 (nuclear factor (erythroid-derived 2)-like 2) to pleomorphic adenoma pathology. The Nrf2-controlled gene system is one of the most critical cytoprotective mechanisms, providing antioxidant responses.

Material/Methods: The study was carried out in pleomorphic adenoma and control parotid gland tissues, investigating gene expression of \textit{NFE2L2}, as well as \textit{KEAP1} (Kelch-like ECH-associated protein 1) and \textit{NQO1} (quinone oxidoreductase), at mRNA and protein (immunohistochemistry) levels. Functional evaluation of Nrf2 system in the parotid gland was evaluated in HSY cells (human parotid gland adenocarcinoma cells).

Results: Pleomorphic adenoma specimens showed cytoplasmic and nuclear Nfr2 expression in epithelial cells, as well as more variable lower Nrf2 level in mesenchymal cells. In the parotid gland, Nrf2 was expressed in cytoplasm of serous, mucous, and duct cells. Nuclear Nrf2 expression was predominantly seen in serous cells, whereas mucous and duct cells were mostly negative. Comparable mRNA levels of \textit{NFE2L2} and \textit{NQO1} genes and significantly higher expression of \textit{KEAP1} in pleomorphic adenoma were seen. HSY cell incubation with oltipraz demonstrated significant elevation of \textit{NFE2L2} after 24 and 48 hours of stimulation, whereas \textit{NQO1} was elevated, but significantly only after 24 hours, and \textit{KEAP1} expression remained unchanged.

Conclusions: Summarizing both \textit{in vitro} and \textit{in vivo} observations, it can be stated that Nrf2 may play a role in the pathology of pleomorphic adenoma.

MeSH Keywords: Adenoma, Pleomorphic • NF-E2-Related Factor 2 • Salivary Gland Diseases

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MOLECULAR BIOLOGY

Background

Pleomorphic adenoma (benign mixed tumor) represents 45% to 74% of all salivary gland tumors and 65% of them occur in the parotid gland. Histologically, pleomorphic adenomas are characterized by the presence of both epithelial and mesenchymal elements, with marked morphological diversity. It is generally accepted that the tumor originates from stem cells or reserve cells of intercalated ducts with further epithelial and mesenchymal cell differentiation [1].

The etiology of pleomorphic adenoma is unknown. Cytogenetic analysis of salivary gland tumors has revealed chromosomal translocations at the breakpoints 8q12, 3p21, and 12q13-155, corresponding to the PLAG1,6 β-catenin7, and HMGIC8 genes, respectively [2–5]. Novel candidate regions on 8p23.1pter, 9p, 10q25.1q25.3, and 11q24qter, with respective candidate genes of MCPH1, ANGPT2, TNKS, PINXI; p15, p16; MXI1, CASP7 and TBRG1, CHEK1, were proposed by Wemmert et al. [6]. Recently, evidence has emerged suggesting that miRNAs may play a profound role in pleomorphic adenoma tumorigenesis, with most elevated levels of hsa-miR-140-5p, hsa-miR-99b, and hsa-miR-140, and the most decreased of hsa-miR-20b, mmu-miR-291-3p, and hsa-miR-144 [7]. Other dysregulated proteins potentially associated with pleomorphic adenoma are human b-defensin-1 [8] and IGF-1 [9].

Despite numerous findings, the pathophysiology of pleomorphic adenoma is still not well established. Therefore, further studies on mechanisms involved in tumorigenesis are needed for a better definition of factors involved in development and progression of those tumors.

The Nrf2-Keap1 (nuclear factor (erythroid-derived 2)-like 2 – Kelch-like ECH-associated protein 1) system is one of the most critical cytoprotective mechanisms. Keap1 is an essential regulator of Nrf2 activity. Under normal conditions, Nrf2 is constantly ubiquitinated through Keap1 in the cytoplasm and degraded in the proteasome. Upon exposure to electrophiles or ROS (reactive oxygen species), Keap1 is inactivated and Nrf2 is stabilized, and consequently it translocates into the nucleus and activates transcription of detoxifying enzymes and antioxidant proteins [10]. It was found that knockout (nrf2−/−) mice display low basal and/or inducible expression of cytoprotective genes (e.g., glutathione S-transferase, thioredoxin reductase, heme oxygenase, metallothionein 1, superoxide dismutase, NAD(P)H quinone reductase 1) in a variety of tissues, and were characterized by an enhanced susceptibility to toxicities associated with various xenobiotics and environmental stresses [11]. Animal studies and human data supports observations from knockout (nrf2−/−), showing that Nrf2 is an important player in pathologies related to oxidative stress, including tumorigenesis [12]. However, recent studies have also demonstrated that Nrf2 promotes survival of not only normal cells but also cancer cells, protecting cancer cells from oxidative insults (from chemotherapy and radiotherapy), creating an environment conducive for cell growth [13]. Nrf2 was identified to regulate the antioxidant response by controlling the expression of genes bearing an ARE (antioxidant response element) in their regulatory regions, such as NQO1 (NAD(P)H quinone oxidase 1). NQO1 catalyzes the two electron reductive metabolism and detoxification of endo- and exogenous compounds, participating in defense against intracellular oxidative stress by scavenging superoxide, maintaining the reduced form of endogenous antioxidants as well as in drug metabolism. NQO1 is also considered to be a reliable reporter of Nrf2 transcriptional activity [14].

Nrf2 mRNA expression findings as well as immunohistochemical evaluation of the human parotid gland revealed cytosolic Nrf2 expression in striated duct cells as well as within myoepithelial cells [15]. Our previous study also revealed constitutive Nrf2 expression, mainly in cytoplasm of intralobular striated duct cells and its induction by specific Nrf2 inducer - oltipraz in rat parotid gland [16].

Therefore, it is important to characterize different types of neoplastic pathologies for Nrf2 expression in order to better understand its involvement in tumorigenesis. In the present study, Nrf2 contribution to pleomorphic adenoma pathology was investigated.

Material and Methods

Tissue specimens

Tissue specimens were sampled from 14 patients, aged from 49 to 66 years (8 females, 6 males) diagnosed with pleomorphic adenoma. From each patient a neoplastic tissue as well as tissue from healthy part of the parotid gland were dissected. A part of fresh specimens sampled was immediately preserved in RNAlater (Applied Biosystems, USA) for RNA expression analysis and adjacent tissue was embedded in formalin for immunohistochemistry. The study protocol was approved by our local ethics committee, and all patients gave informed consent.

Cell culture

Human parotid gland adenocarcinoma cells (HSY) (provided by Dr. M. Sato, Tokushima University, Japan) were seeded in 24-well tissue culture plates, 5×10⁴ per well into DMEM medium (Sigma, Germany), supplemented with 10% FBS (Invitrogen, USA) and 0.4% streptomycin/penicillin (Sigma, Germany), and incubated at 37°C in a humidified incubator supplied with 5% CO₂. After 24 h, medium was replaced with DMEM medium without FBS, containing 0.5% BSA and oltipraz (dissolved in DMSO) at a concentration of 100 μM, as well as in control cells with
a respective medium with DMSO (without oltipraz). Oltipraz was used as a model agent because it has a robust effect on Nrf2. Likewise, M2 metabolite from oltipraz was shown to be a strong in vivo activator of Nrf2 [17]. After the subsequent 24/48 h of incubation, medium was decanted, and RNA was immediately extracted from the cells using RNAqueous Micro Kit (Ambion, USA). Afterwards, isolated RNA was used for qRT-PCR analysis. The experiments were performed in triplicate.

Quantitative real-time PCR analysis

Total RNA was extracted from 20-mg tissue specimens by means of Direct-zol RNA MiniPrep Kit (Zymo Research Corporation, USA). Subsequently, cDNA was prepared from 500 ng of total RNA in 20 μl of reaction volume, using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) with oligo-dT primers, according to the manufacturer’s instructions. Quantitative expression of the following genes, using two-step reverse transcription PCR was measured: NFE2L2, KEAP1 and NQO1 (a reliable reporter of Nrf2 transcriptional activity), together with house-keeping endogenous control genes: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PPIA (cyclophilin A) and GUSB (beta-glucuronidase). qRT-PCR was performed in ViIA™ 7 Real Time PCR System (Life Technologies, USA), using pre-validated Taqman Gene Expression Assays, TaqMan® Fast Advanced Master Mix (Applied Biosystems, USA) and 1.5 μl of cDNA for each reaction mix of 15 μl. Each sample was analyzed simultaneously in two technical replicates, and mean C\(_T\) values were (+++) Very strong expression; (+++ strong expression; (+) weak expression; (–) lack of expression. The percentage of Nrf2 positive cells was counted manually (from 10 consecutive high-power fields).

### Table 1. Immunolocalization and immunoexpression of Nrf2 in pleomorphic adenoma and parotid gland.

| Tissue                  | Serous cells       | Mucous cells      | Duct cells      |
|-------------------------|--------------------|------------------|----------------|
|                         | (+++) (>90% cells) | (+++) (50% cells)| (++++) (>90% cells)|
| Parotid gland           |                    |                  |                |
| Cytosolic               | Positive (>90% cells) |                |                |
| Nuclear                 | Positive (>90% cells) |                |                |
| Pleomorphic adenoma     |                    |                  |                |
| Cytosolic               | (+++) (>95% cells)  | (+++) (>95% cells)|                |
| Nuclear                 | Positive (>95% cells) |                |                |

(++) Very strong expression; (+++ strong expression; (+) weak expression; (–) lack of expression. The percentage of Nrf2 positive cells was counted manually (from 10 consecutive high-power fields).

**Figure 1.** Immunohistochemical expression of Nrf2 in human parotid gland (A) and pleomorphic adenoma (B) (magnification 200x, scale bars in the respective figures). (A) Parotid gland: positive nuclear staining and very strong and strong cytoplasmic expression of Nrf2 in serous cells (yellow arrows); mucous cells (red arrows) demonstrate weak cytoplasmic expression of Nrf2. (B) Pleomorphic adenoma: positive nuclear and cytoplasmic Nrf2 expression in epithelial cells (black arrows), only single cells are negative (green arrows); mesenchymal cells (blue arrows) demonstrate moderate cytoplasmic and nuclear expression of Nrf2.
used for further analysis. Calculations were performed using the ΔΔCt relative quantification method, using integrated instrument software (Life Technologies, USA). The thresholds were set manually to compare data between runs, and Ct values were extracted. All Ct values for each sample were normalized to the geometric mean value obtained for three control genes, processed in the same run. Fold change between groups was calculated from the means of the logarithmic expression values.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded 5-μm sections of specimens from healthy parotid gland tissue as well as from pleomorphic adenoma were deparaffinized, rehydrated and immersed in pH 9.0 buffer. Heat-induced antigen retrieval was performed in a pressure cooker (Pascal, Dako, Denmark) at 120°C for 3 minutes. Slides were incubated with primary rabbit polyclonal anti-Nrf2 antibody (ab31163, Abcam, USA, dilution 1: 50) for 30 minutes at room temperature and immunostained with a Dako Envision + kit for 30 minutes, AEC + as a chromogen and hematoxylin as counterstain. Normal mouse immunoglobulins were substituted for primary antibodies as negative controls. In establishing the method, placenta and kidney were used as positive control tissues (with known Nrf2 expression). A semi-quantitative analysis was performed, with the following grading system: (+++) very strong expression, (++) strong expression, (+) weak expression, (−) lack of expression. The percentage of Nrf2 positive cells was counted manually (from 10 consecutive high power fields).

Results

Expression of Nrf2 was observed in human parotid salivary gland and pleomorphic adenoma tissue, both at mRNA and protein level (evaluated by immunohistochemistry). In parotid gland, Nrf2 was expressed in serous, mucous, and duct cells, especially in cytoplasm of these cells. Nuclear expression was predominantly seen in serous cells, whereas mucous and duct cells were mostly negative for Nrf2 nuclear expression (Table 1 and Figure 1). In pleomorphic adenoma, epithelial cells and mesenchymal cells both showed Nrf2 expression, mostly cytoplasmic, but more differentiated for the expression level in mesenchymal cells, where very strong as well as weak expressions in particular cells were detected (Table 1 and Figure 1). Quantitative expression at mRNA level showed comparable levels of NFE2L2 and NQO1 genes (but by 23% and 33% higher in pleomorphic adenoma tissue for NFE2L2 and NQO1, respectively).
respectively), and significantly higher expression of KEAP1 in pleomorphic adenoma tissue in comparison to healthy parotid gland (by 60%, p<0.05) (Figure 2).

The HSY cell study revealed an effect of specific Nfr2 inducer, i.e., oltipraz. Incubation of HSY cells with oltipraz containing medium demonstrated significant elevation of NFE2L2 both after 24 and 48 hours of stimulation. The expression of Nfr2 controlled gene, i.e., NQO1 was elevated, but significantly only after 24 hours. Level of KEAP1 remained unchanged under 24- or 48-hour oltipraz stimulation (Figure 3).

Discussion

The pathology of pleomorphic adenoma is not well defined. It is proposed that the tumor originates from stem cells or reserve cells of intercalated ducts with further epithelial and mesenchymal cell differentiation. This pool of stem cell or a reserve cell population is a reservoir of cells for maintaining morphological and functional integrity or may give an origin for neoplasia. The semipotentipotential cell biological hypothesis for tumor induction explains morphological diversity observed in pleomorphic adenoma [1]. However, this concept is not supported by experimental findings. In fact, it has been demonstrated in experimental models that the capacity for cellular proliferation was shown by other cell types in the salivary glands: acinus cells, cells at all levels of ductal segments, and myoepithelial cells, which could proliferate under different physiological and pathological conditions [revised in 18]. Therefore, the trigger mechanisms and other factors implicated in the tumor development and progression still require definition.

The Nfr2-Keap1 signaling pathway is one of the most important cell defense mechanisms. Nfr2 can protect cells and tissues from a wide range of toxins, including carcinogens. It coordinates transcription/function of an array of genes involved in antioxidative responses, including evaluated in the present study quinone oxidoreductase (NQO1) [19]. Nfr2 knockout mice (nfr2−/−) show that Nfr2 protects against chemical carcinogen-induced tumors, i.e., gastric neoplasia after exposure to benzo(a)pyrene [20], bladder tumors upon exposure to N-nitrosobutyl(4-hydroxybutyl)amine [21], skin tumors following exposure to 7,12-dimethylbenz(a)anthracene or 12-O-tetradecanoylphorbol-13-acetate [22], most probably due to its ability to reduce the amount of reactive oxygen species (ROS) and DNA damage in cells. Therefore, it seems that Nfr2-deficient status predisposes to carcinogenesis.

In spite of constant Nrf2 ubiquitination, Nrf2 expression is high in human kidney, skeletal muscle, and lung, moderately expressed in liver, placenta, and heart, and least expressed in brain and pancreas [23]. High expression of Nrf2 is associated with activation of cellular antioxidant and xenobiotic detoxification systems. There is no human data comparing expression level of Nrf2 in salivary glands and other tissues. Our rat study demonstrated similar expression of Nrf2 in liver and parotid gland, but liver levels of Nqo1 and Keap1 were significantly higher than in parotid gland: parotid/liver ratio 0.03 and 0.06, respectively [16].

The present study demonstrated comparable cytosolic expression of Nrf2 in serous and duct cells, and lower in mucous cells in native healthy parotid gland tissue. However, nuclear expression, most closely reflecting Nfr2 functional state, was found to be high only in serous cells. Since Nrf2 operates in nucleus (being in cytoplasm bound to its repressor Keap1), is seems that mucous and duct cells are less effectively protected from oxidative stress than serous cells. The last finding may also reflect higher oxidative stress in serous cells (and if protective mechanisms are not efficient, their higher vulnerability), and the letter observation may implicate lower oxidative stress in mucous and duct cells.

Animal studies demonstrated responsiveness of parotid gland Nfr2-Nqo1 system upon exposure to inducers. Yates et al. reported that salivary gland and small intestine mucosa were characterized by the highest (higher than liver) level of Nqo1 induction (23.6- and 19.8-fold, respectively) by 1-[2-cyano-3,12-dioxygenana-1,9(11)-diene-28-oyl]imidazolide in Nqo1-ARE-Luc reporter mice [24]. Our previous study in rats revealed also that Nfr2 induction by oltipraz produced significant elevation of Nqo1, and no effects on Nrf2, Keap1 and Ugt1a6 expression in parotid gland [16]. Our present study characterized NFE2L2 responsiveness in human parotid gland cells, i.e. HSY (human parotid gland adenocarcinoma cell line) cells, that have an ultrastructure similar to human salivary intercalated duct cells [25], under exposure of gene specific inducer, i.e. oltipraz. The HSY cell study demonstrates that the cells possess adaptive mechanisms responding to specific Nrf2 inducer, i.e., oltipraz. Stimulation of HSY cells resulted in significant elevation in expression levels of both NFE2L2 and NQO1 (an enzyme providing antioxidant activity) genes, but activation of NQO1 gene seem to be somehow transient, as longer (48-hour) co-incubation with oltipraz did not produce marked elevation of the gene expression. Therefore, the expression picture seen in HSY cells may suggest that intensive nuclear expression of Nfr2 seen in serous cells resulted from higher oxidative stress.

Quantitative mRNA study revealed similar levels of NFE2L2 (coding for Nfr2) and NQO1 gene expression in normal parotid gland and pleomorphic adenoma, along with significantly elevated KEAP1 expression in pleomorphic adenoma tissue. This finding may suggest that pleomorphic adenoma may be characterized by reduced Nrf2 function, and thus its coordinated antioxidant system, due to (shown in the present study) elevated KEAP1 (Nrf2 repressor) expression in the tumor tissue. However, cellular distribution is also important for functional...
When pleomorphic adenoma is formed, Nrf2 can promote further Nrf2 transformation in the epithelial component more efficiently than in mesenchymal cells. However, our study is descriptive and gives insight only into Nrf2 expression in parotid gland and pleomorphic adenoma (any functional experiments do not directly support the above hypothesis).

**Conclusions**

Summarizing both in vitro and in vivo observations, it can be stated that Nrf2 may play a role in the pathology of pleomorphic adenoma. Other detailed studies are required to precisely define the role of Nrf2 in the pathogenesis of pleomorphic adenoma.

**Conflict of interest**

All the authors had no conflicts of interest to declare in relation to this article.

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