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Salivary Leptin as a Candidate Diagnostic Marker in Salivary Gland Tumors

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BACKGROUND: Since the discovery of autonomous leptin production in salivary glands, very few studies have reported on the physiological or pathological meaning of this particular cytokine in saliva. The aim of this study was to investigate the expression of leptin and its receptors Ob-Ra and Ob-Rb in parotid salivary gland tumors, with particular regard to a potential use of leptin as a tumor marker.

METHODS: Parotid tissue samples from healthy individuals (n = 31) and tumor patients (n = 97, including tissue samples from pleomorphic adenomas, adenolymphomas, basal cell adenomas, and diverse carcinomas) were analyzed by use of ApoTome-technique microscopy, immunohistochemistry, immunofluorescence, immunoblotting, and quantitative real-time PCR. Salivary and plasma leptin concentrations were measured by using ELISA. Ultrasound was used to determine tumor size before surgery.

RESULTS: In all salivary gland tumors leptin was expressed in much higher amounts than in healthy parotid tissues. The cytokine was not imported from the blood but actively produced by the tumors. Immunoblotting results indicated that leptin was present as oligomers in salivary glands. Furthermore, the examined tumors overexpressed the receptor isoforms Ob-Ra and Ob-Rb. Measured leptin concentrations in mixed saliva samples were significantly increased in patients with parotid tumors [mean (SD) 673 (484) pg/mL in pleomorphic adenomas, 679 (465) pg/mL in adenolymphomas, and 880 (618) pg/mL in carcinomas] vs controls [125 (36) pg/mL] ($P < 0.001$).

CONCLUSIONS: This is the first study to show that the analysis of salivary leptin in mixed saliva samples may allow preoperative differentiation between tumor patients and healthy individuals.

Saliva contains various endogenous substances, and the analysis of certain salivary hormones is an accepted alternative to plasma analysis *(1)*. Salivary steroid concentrations reflect the non–protein-bound, physiologically active part of the blood concentration *(2)*. Amines and peptides such as melatonin and insulin enter the saliva by either diffusion or active transport. For these hormones, excellent correlations were seen between saliva and blood concentrations *(3–5)*.

In the evaluation of salivary gland masses, inflammatory cytokines such as interleukin 8 *(6)* and endothelial growth factor *(7)* may hold interest as potential tumor markers. One of the more interesting cytokines expressed and secreted by salivary glands is leptin *(8, 9)*. Leptin was originally reported to be a regulator of food intake and energy expenditure *(10)*. However, it has since gained increasing attention as a factor involved in tumorigenesis *(11, 12)* and metastasis *(13, 14)*. This cytokine is present in the gastrointestinal tract, where it meets criteria of endocrine and exocrine secretion *(15)*. Salivary glands express leptin and its receptors in acini and intralobular duct cells *(8, 16, 17)*, and saliva from healthy individuals has been found to contain leptin *(18, 19)*.

One demonstrated effect of salivary leptin is the enhancement of proliferation of oral mucosal cells *(20)*. However, a potential impact of salivary leptin on tumorigenesis has not yet been investigated. The fact that leptin is associated with tumors and is produced by salivary glands and secreted into saliva prompted us to investigate the expression of leptin and its various receptors in human salivary gland tumors.

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Materials and Methods

All samples were obtained with patient consent and in accordance to the guidelines of the Ethical Committee of the University of Erlangen-Nuremberg.

TISSUE SAMPLES

Human parotid gland tumor samples were obtained from 97 patients (54% male) age 38–85 years [mean (SD) 62 (17) years]. Tissues of healthy parotid glands were obtained from 31 patients (55% male) age 32–83 years [mean (SD) 58 (15) years] undergoing surgery for other indications. One portion of each tissue sample was fixed in phosphate buffered paraformaldehyde (4%) overnight, dehydrated, and embedded in paraffin; other portions were stored in RNAlater solution (Qiagen) according to manufacturer's protocol or directly cryopreserved and stored at −80 °C.

SALIVA SAMPLES

Mixed unstimulated saliva samples were obtained from 77 tumor patients [58% male, 24–89 years, mean (SD) 57 (15) years, 58% smokers] 1 day before surgery, 2 to 3 h after breakfast, and before lunch. Samples were collected by use of polyester Salivettes® (Sarstedt). Samples were immediately centrifuged (10 min, 1100*g*) and stored at −80 °C. In the same manner, control saliva samples were obtained from 22 healthy individuals [59% male, age 24–80 years, mean (SD) 52 (17) years, 50% smokers].

Samples from patients with salivary gland stones [n = 11, age 14–65 years, 42 (15) years, 64% smokers] and cysts [n = 4, age 47–86 years, mean 68 (15) years, 25% smokers] provided an additional control group. All groups were matched in male/female ratio, mean age, and mean body mass index (BMI).

SERUM SAMPLES

Whole blood samples were collected from 60 tumor patients and 18 healthy individuals [25–82 years, mean 57 (17) years] using S-Monovetten® (Sarstedt) according to manufacturer's instructions at the same times as saliva samples. All samples were centrifuged immediately (10 min, 1100*g*), and the supernatant serum was stored at −80 °C until analysis.

PRIMARY ANTIBODIES

For immunohistochemistry and Western blots we used purified rabbit polyclonal antibodies to antihuman leptin (SC-842) and purified goat polyclonal antibodies directed against the C-terminus of the short form of leptin receptor of mouse origin [both from Santa Cruz Biotechnology *(21)*]. For repeated Western blots, different rabbit antihuman leptin antibodies were chosen (Sigma-Aldrich; Biozo; Mediagnost). For immunofluorescence we used serum from New Zealand White rabbits *(22)* immunized with recombinant human leptin (Mediagnost); serum samples were purified with protein A Sepharose (Amersham).

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Consecutive paraffin tissue sections (3 μm) were dewaxed in xylene and rehydrated in graded alcohol series, and then antigen unmasking was carried out at 90 °C for 20 min (Target-Retrieval-Buffer, Dako). Nonspecific binding was inhibited by covering slides for 30 min with 5% normal serum of the species from which the secondary antibody was raised, diluted with PBS.

For leptin staining, sections were incubated overnight with primary antibodies diluted 1:80 in 5% normal goat serum; in negative controls, primary antibodies were omitted. Goat-antirabbit secondary antibodies, readily alkaline–phosphatase-conjugated and diluted 1:300 in 5% normal goat serum, were applied for 30 min (Vector Laboratories).

Leptin receptors were stained by using primary antibodies, diluted 1:100 in 5% normal horse serum, and incubated overnight. Alkaline–phosphatase-conjugated horse-antigoat secondary antibodies (Vector Laboratories), diluted 1:300 in 5% normal horse serum, were incubated for 30 min. Antigens were stained with Vector®-Red substrate reagent for alkaline phosphatase (Vector Laboratories), and counterstained with hematoxylin.

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Frozen sections (5 μm) were kept at room temperature for 15 min before treatment with methanol at −20 °C for 10 min. After 20 min of blocking with Roti-ImmunoBlock (Carl Roth), primary antibodies, diluted 1:100 in Roti-ImmunoBlock, were applied for 90 min. In negative controls, antibodies were omitted. Secondary antibodies, Cy-3–labeled donkey-antirabbit IgG (Dianova), diluted 1:400 in Roti-ImmunoBlock (Carl Roth), were used in combination with 4′,6-diamidino-2-phenylindole (10 μg/L) for 45 min.

WESTERN BLOTS

For each tissue sample, 30–50 mg was homogenized (T25 Basic, IKA Labortechnik) in 1.0 mL lysis buffer [50 mmol/L Tris, pH 7.4; 300 mmol/L NaCl; 10 mmol/L EDTA; 1% NP40; 0.1% Triton-X100; 0.1% SDS; and protease inhibitor cocktail Complete® (Boehringer)]. Samples were incubated at 4 °C for 2 h and centrifuged at 16000*g* for 1 h. Protein concentration of the supernatant was analyzed with bicinchoninic acid assays (Pierce Biotechnology), and aliquots were stored at −80 °C until use.

After denaturation (5 min at 90 °C with SDS loading buffer including mercaptoethanol), 50 μg of lysate

was applied to each lane, separated by SDS-PAGE on 16% gels, and transferred to nitrocellulose membranes (Protran-BA-83, Schleicher & Schuell). Ponceau-S staining served as an additional loading control. For detection, primary antibodies 1:200 in RotiBlock (Carl Roth) were used. Horseradish peroxidase–conjugated goat-antirabbit IgG (Bio-Rad Laboratories), 1:5000 in RotiBlock, was applied for 60 min, followed by electrochemiluminescence for 60 s. Signals were detected by use of a LumiImager-F1 (Roche).

For immunoblotting of saliva, identical volumes of samples were pooled from each group (n = 20 for healthy individuals, n = 20 for pleomorphic adenomas, n = 20 for adenolymphomas, n = 10 for carcinomas, and n = 8 for cysts). For each pool, 400 μL saliva was incubated for 2 min with 20 μL Stratagene Resin® (Agilent Technologies) and centrifuged for 1 min with 1000*g*. SDS loading buffer including mercaptoethanol was added to each pellet, and charges were incubated at 90 °C for 5 min before loading on 16% SDS-PAGE gels. Loading controls and detections were performed as described above.

ELISA

We determined salivary and serum leptin concentrations by using a highly specific ELISA (Leptin-Duo-Set, R&D-Systems) with a sample volume of 100 μL. The sensitivity of the system was 7.8 ng/L, with a CV of 3% in intraassay and 3.5%–5.4% in interassay analyses. Saliva standards were diluted in a low-protein matrix to adjust the system to the protein content of saliva (R&D-Systems). For serum standards, a high-protein matrix for serum analysis was used for dilution (R&D-Systems). All samples were analyzed as duplicates. Salivary leptin concentrations were additionally related to each patient's BMI.

QUANTITATIVE REAL-TIME PCR

We isolated total RNA from healthy, pleomorphic adenoma, and adenolymphoma tissue samples using illustra RNAspin (GE-Healthcare) according to manufacturer's instructions, including genomic DNA digestion. We used 1 μg of total RNA, determined by photometric analysis, for reverse transcription (Qiagen), obtaining 20 μL of final volume.

For all real-time PCR reactions, solutions provided by a qPCR-Core-Kit for SYBR-Green I – No Rox (Eurogentec Deutschland) and iCycler real-time PCR devices (Bio-Rad) were used. Each amplification step consisted of denaturation, annealing, and elongation at optimized temperatures and primer concentrations (see Supplemental Table 1 available at http://www.clinchem.org/content/vol55/issue5 with the online version of this article). All samples were analyzed as triplets; their arithmetic mean was compared with 7 triplets of standardized leptin cDNA amplified within the same run. We used water as a negative control. For determination of concentrations, iCycler software (iCycler IQ, Optical-System-Software, version 3.0a, Bio-Rad) was used. For normalization, we used a GeNorm software algorithm *(23)*, which selected tubulin, alpha 1a (*TUBA1A*)[3] and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as most suitable of 3 housekeeping genes [*GAPDH*, *TUBA1A*, and beta-2-microglobulin (*B2M*)]. To exclude false results from adipocyte-produced leptin-mRNA, adiponectin ApM-1 [adiponectin, C1Q and collagen domain containing adiponectin (*ADIPOQ*)] was chosen as a specific adipocyte marker. Housekeeping gene–normalized cDNA concentrations of leptin, Ob-Ra, and Ob-Rb were related to housekeeping gene–normalized ApM-1 cDNA levels of the corresponding tissue sample.

ULTRASOUND-GUIDED SIZING OF TUMORS

To determine whether leptin concentrations in saliva were influenced by the tumor size, ultrasound-guided sizing of each tumor was carried out before surgery (GE-Healthcare). The extension in all 3 dimensions was determined, and the volume was calculated and correlated to salivary leptin concentrations.

STATISTICAL ANALYSIS

We carried out statistical calculations using the Kruskal–Wallis test and Dunn's Multiple-Comparison test. Effects of smoking on salivary leptin levels were analyzed with unpaired *t*- or Mann–Whitney tests. Differences with a *P*-value <0.05 were considered as significant.

Results

LEPTIN IN TISSUE SAMPLES

All methods showed a strong increase of leptin within tumors compared with healthy tissues.

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Leptin. All entities of salivary gland tumors examined showed intense and equal staining of leptin, with at least 95% of tumor cells being leptin positive. Leptin was present in intercalated, striated, and intralobular ducts as well as in basal parts of the acini in healthy glands (Fig. 1A), but its expression was not limited to defined structures in tumors. Cytoplasmatic staining

[3] Human genes: *TUBA1A*, tubulin, alpha 1a; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *B2M*, beta-2-microglobulin; *ADIPOQ*, adiponectin, C1Q and collagen domain containing.

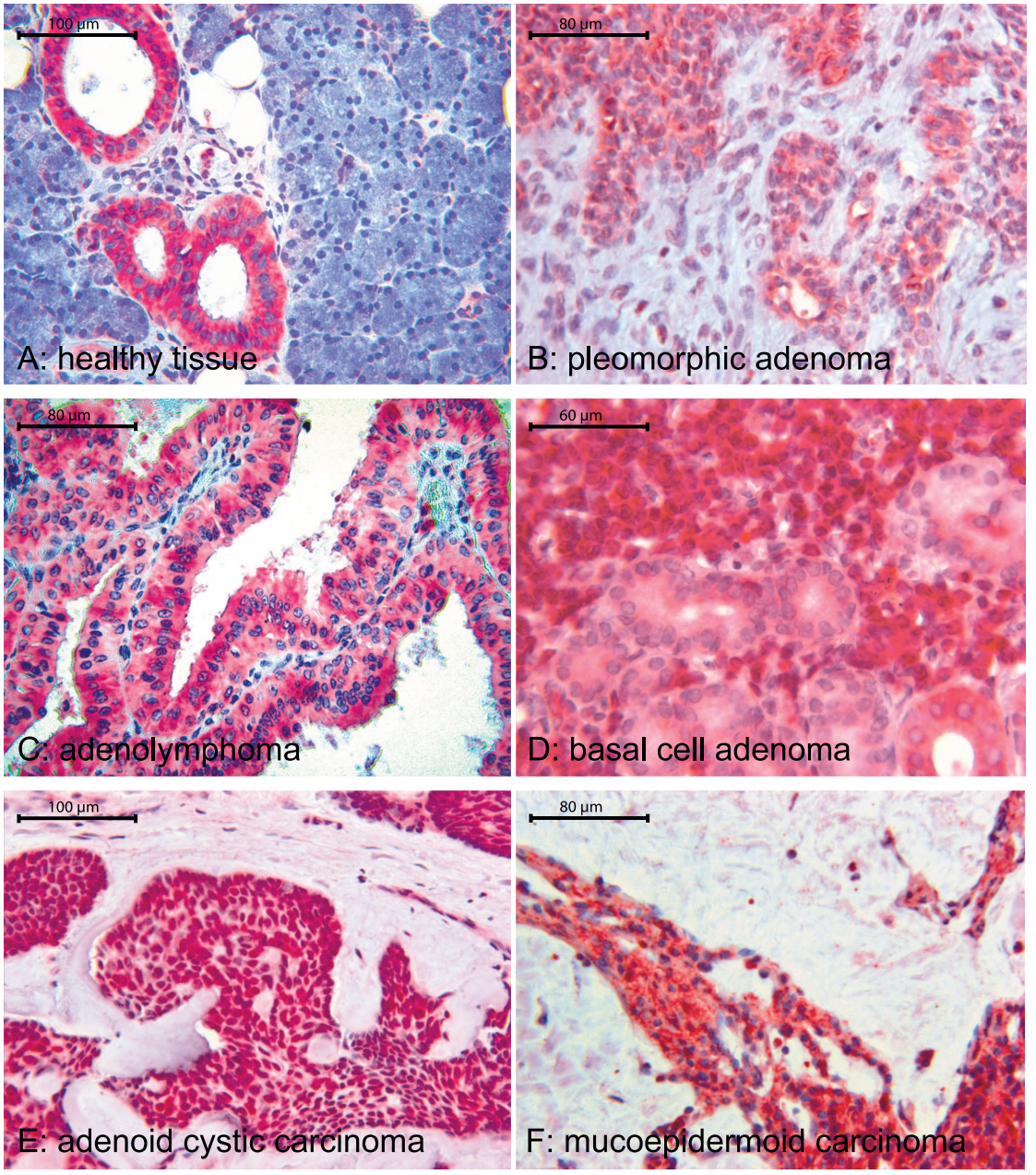

Fig. 1. All examined types of parotid gland tumors showed intense immunohistochemical staining for leptin (red) throughout the whole tumor.

was predominant in all tissues. Pleomorphic adenomas displayed cribriform-arranged leptin-positive cells amidst a mucous matrix (Fig. 1B). The expansion of leptin-positive cells was limited by the tumor capsule. In adenolymphomas, epithelial cells were much more intensely stained for leptin (Fig. 1C) than lymphatic areas of the tumors, where the antigen was also present (not shown). In basal cell adenomas (Fig. 1D), intensely leptin-stained cells, arising from the outer stratum of the double-layer epithelium of intercalated ducts, were spread throughout the tumor, representing the proliferating fraction.

Bridging of leptin-positive cells surrounded by a mucilaginous matrix was found in mucoepidermoid-

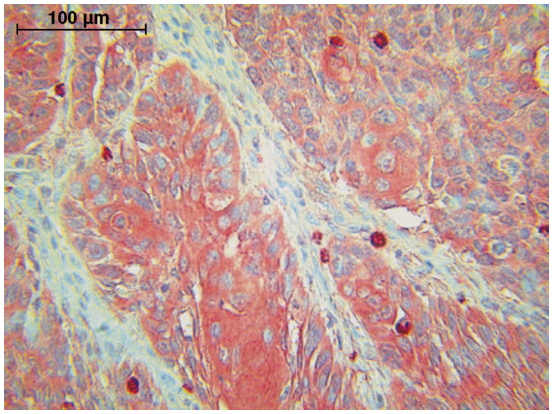

Fig. 2. Representative immunohistochemical staining for leptin receptors (red) in a pleomorphic adenoma of the parotid gland.

Staining does not differentiate between the receptor isoforms, and increased leptin receptor levels can be found in all tumor entities.

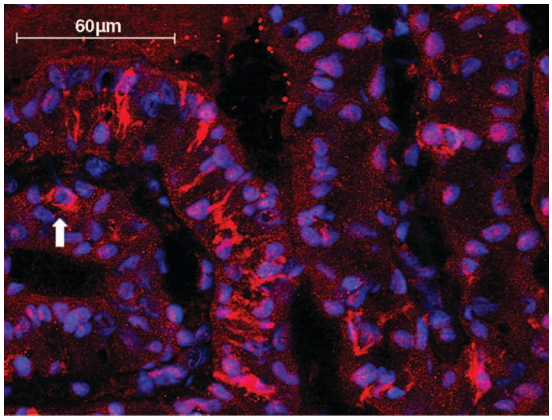

Fig. 3. Immunofluorescence staining for leptin.

Leptin (red) is present in cytoplasmatic granules. Staining is most intense at the apical cell poles of the epithelium, as shown here in a representative adenolymphoma. Nuclei are stained in blue (with 4′,6-diamidino-2-phenylindole).

carcinomas (Fig. 1F). In adenoid cystic carcinomas, staining was observed in cells that form the typical structures of these tumors (Fig. 1E). Squamous cell carcinomas demonstrated cytoplasmatic staining for leptin in densely aggregated tumor cells, and adenocarcinomas showed a more clod-like leptin-positive cell arrangement (both not shown).

Leptin receptors (OB-Ra and OB-Rb). In healthy tissues the different leptin receptor isoforms were expressed in a similar pattern to leptin, only in intercalated, striated or intralobular ducts and adipocytes, whereas in all tumors of the parotid gland that we examined, intense leptin receptor–positive cells were present throughout the whole tumor (Fig. 2). For a representative pleomorphic adenoma, however, we found areas of weakly stained cells surrounded by cells that were strongly positive for leptin receptors, shown in Fig. 2. Single intensely stained cells, presumably leukocytes, were dispersed throughout both healthy and tumorous parotid gland tissues.

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When examined by use of the ApoTome-technique, immunofluorescence revealed leptin-positive cytoplasmatic vesicles, shown in Fig. 3 for a representative adenolymphoma. In several cases, immunoreactive granules could be found in higher density next to the nucleus (Fig. 3, arrow), presumably depicting the endoplasmic reticulum.

IMMUNOBLOTTING OF SALIVARY GLAND TISSUES

Western blot analysis revealed an increased amount of leptin in salivary gland tumor tissues of all analyzed entities compared with healthy salivary glands (Fig. 4A). Surprisingly, the most intense bands in all tumors as well as in all healthy tissues did not show up at a molecular mass of 16 kDa as expected but were localized at mass levels which seemed to represent multiples of the monomeric molecular mass (Fig. 4B, right). This pattern was also seen with recombinant standard leptin (Bachem), for which the most intense band was located at 16 kDa, with a weaker band that seemed to represent a dimeric structure with double molecular mass (Fig. 4B, left). We were able to reproduce this outcome with 4 different antileptin antibodies across all other salivary gland tumor lysates examined (not shown). Fig. 4A shows the most intense bands, located at 32 kDa; their intensity is also representative for the additional bands of each lysate.

IMMUNOBLOTTING OF POOLED SALIVA

Immunoblotting of pooled saliva confirmed the prior findings from Western blots of gland tissue lysates. Saliva samples from patients with salivary gland tumors showed a much higher amount of leptin compared with samples from healthy individuals or persons suffering from cysts. In contrast with tissue immunoblots, the 16-kDa bands were most intense in saliva samples. Additional bands indicating dimeric structures were observed for concentrated, pooled saliva samples across all tissue groups, as shown in Fig. 4C, particularly for the tumor groups.

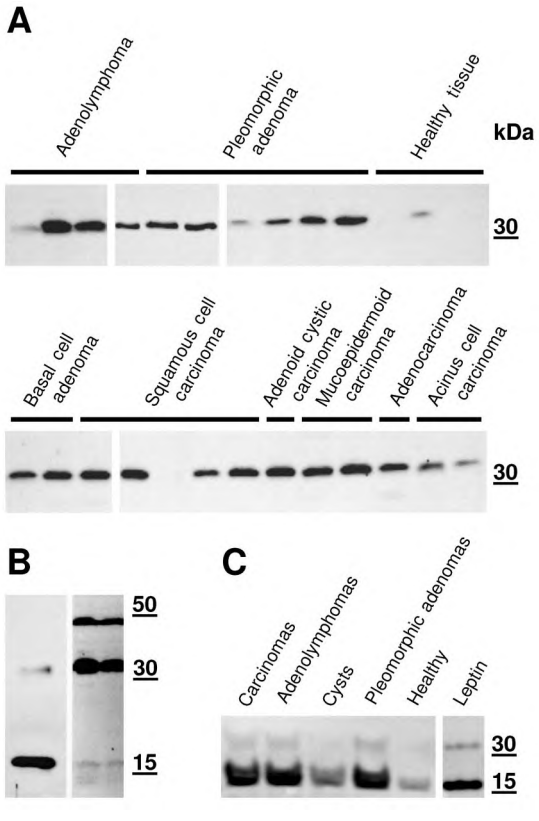

Fig. 4. Immunoblots of leptin in tissue from healthy persons and from patients with various tumor types of the parotid gland.

(A), Tissue lysates from all 8 tumor types had noticeably increased amounts of leptin compared to healthy tissues; 32-kDa bands are shown here. (B), Recombinant human leptin presenting with an intense band at 16 kDa and a weaker band at 32 kDa (left), and leptin oligomers (B, right), from 2 representative adenolymphoma tissue lysates. (C), Concentrated salivas pooled by tissue type and recombinant human leptin.

QUANTITATIVE REAL-TIME PCR

Adiponectin (ApM-1). We focused on using parotid tissue exclusively for PCR. Normal parotid tissue contains adipocytes distributed throughout the tissue. However, tumors of the parotid glands have no or very few adipocytes. Quantitative real-time PCR analysis of healthy tissues (n = 19), pleomorphic adenomas (n = 13), and adenolymphomas (n = 13) revealed that after normalization to 2 housekeeping genes, ApM-1–mRNA levels were significantly lower in tumors than in healthy tissues ($P < 0001$, not shown). This result confirms prior observations with immunohistochemical and hematoxylin and eosin staining showing adipocytes were nearly or completely absent within the tumorous areas of the glands.

Leptin. After normalization to 2 housekeeping genes, leptin-mRNA levels of healthy and tumor tissues were adapted to normalized ApM-1-mRNA concentrations. This procedure was necessary owing to the presence within healthy tissue of adipocytes, which contribute to leptin-mRNA production just as the parenchymal cells do. By contrast, adipocytes are nearly or completely absent in tumors. Leptin-mRNA production was found to be increased in pleomorphic adenomas (n = 13) up to 7-fold and in adenolymphomas (n = 11) up to 4-fold on average compared with controls (n = 16; $P < 0.05$, Fig. 5A).

Leptin receptors (OB-Ra and OB-Rb). Because adipocytes also express leptin receptors, all measured leptin-receptor mRNA levels were adapted, like leptin, to ApM-1–mRNA concentrations. Normalization of Ob-R–mRNA levels to 2 housekeeping genes and ApM-1-mRNA levels revealed that leptin receptor production is enormously increased in the 2 benign entities of parotid gland tumors examined. We used samples of healthy parotid glands with their Ob-R–mRNA levels, normalized to 2 housekeeping genes and ApM-1-mRNA levels, as controls (n = 18 for Ob-Ra, n = 14 for Ob-Rb). Whereas mRNA production of Ob-Ra, the truncated isoform of the receptor (Fig. 5B), was increased on average 74-fold in pleomorphic adenomas (n = 13) and 125-fold in adenolymphomas (n = 13), the production of the functional receptor Ob-Rb–mRNA (Fig. 5C) was increased up to 42-fold in pleomorphic adenomas (n = 11) and up to 72-fold in adenolymphomas (n = 13).

LEPTIN IN SALIVA AND SERUM SAMPLES

Leptin concentrations were significantly higher ($P < 0.001$) in saliva of tumor patients (Fig. 6A; n = 29 for pleomorphic adenomas, n = 34 for adenolymphomas, n = 14 for carcinomas) than in healthy individuals (n = 22). By contrast, salivary leptin concentrations in patients suffering from parotid gland stones (n = 11) and cysts (n = 4) were not found to differ significantly from healthy controls ($P > 0.64$). Mean (SD) salivary leptin concentrations in patients with pleomorphic adenoma patients were 673 (484) ng/L vs 679 (465) ng/L in patients with adenolymphoma. In carcinoma patients mean salivary concentrations were 880 (618) ng/L. In healthy individuals, on the contrary, mean concentrations were 125 (36) ng/L.

To evaluate whether the high concentrations of leptin in saliva were caused by obesity, we also determined leptin concentrations in serum samples (n = 18 for controls, n = 18 for pleomorphic adenomas, n = 10

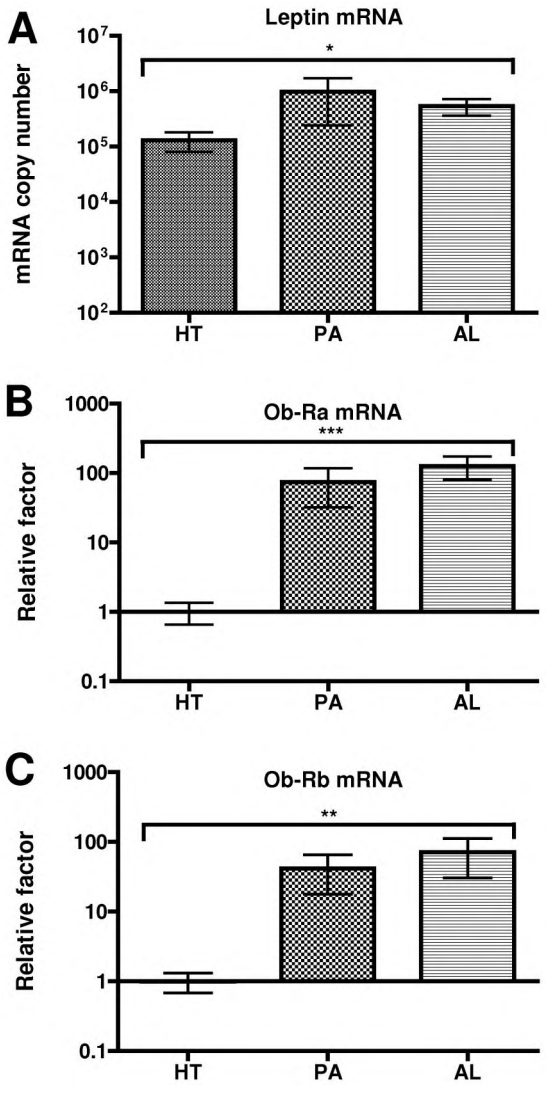

Fig. 5. (A), Normalized leptin-mRNA copy numbers in healthy tissue (HT), pleomorphic adenoma (PA), and adenolymphoma (AL) revealed the significantly increased leptin-mRNA copy number within tumors (*$P < 0.05$).

(B), For Ob-Ra, both tumors showed significantly increased mRNA expression for the truncated receptor isoform, on average up to 74-fold in pleomorphic adenomas and 125-fold in adenolymphomas (***$P < 0.001$). (C), For Ob-Rb, mRNA levels were significantly increased up to 42-fold in pleomorphic adenomas and 72-fold in adenolymphomas (**$P < 0.01$).

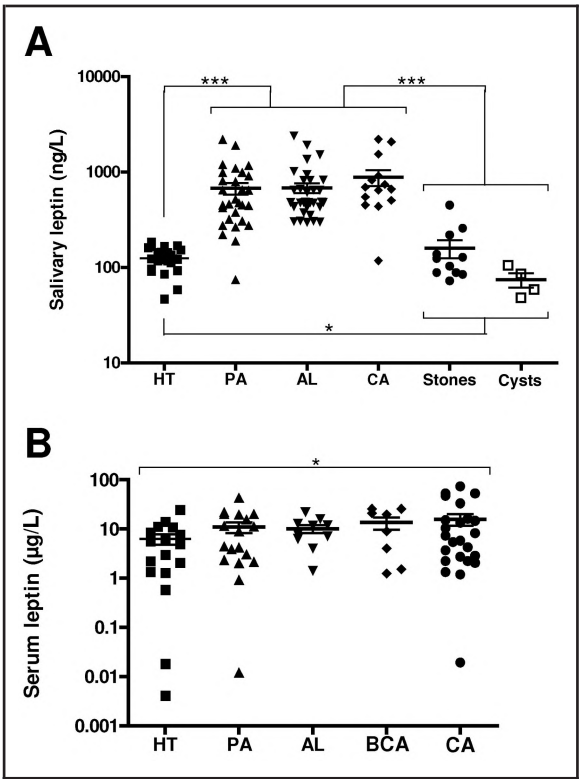

Fig. 6. (A), Salivary leptin concentrations (ng/L).

Averaged and related to healthy tissues (HT), pleomorphic adenomas (PA), and adenolymphomas (AL) show increases up to 5.4-fold and carcinomas up to a 7-fold. Mean concentrations in tumors were statistically different from those in stones and cysts, whereas concentrations stones and cysts did not differ significantly from those in healthy controls (***$P < 0.001$; *$P > 0.64$). CA, carcinoma. (B), Serum leptin concentrations (μg/L). No significant mean differences were observed between any of the groups of patients (*$P > 0.36$). BCA, basal cell adenoma.

for adenolymphomas, n = 8 for basal cell adenomas, n = 24 for carcinomas). No statistically significant differences in serum leptin concentrations were observed between these groups ($P > 0.36$, Fig. 6B). In addition, we related the measured salivary leptin concentrations to each patient's BMI. The results were nearly the same as those obtained without normalization of salivary leptin levels to BMI. However, by normalization to BMI, mean levels in healthy individuals were found to differ from those in patients with cysts ($P < 0.05$), but not from those in patients suffering from stones ($P > 0.05$), enabling us to differentiate between stones and cysts ($P < 0.01$).

Smoking behavior did not have significant effects on salivary leptin concentrations. Within the group of healthy individuals, salivary leptin concentrations of smokers [n = 11, 115 (9) ng/L] were not found to be significantly different from those of nonsmokers (n =

11, 141 (13) ng/L; $P > 0.12$). The same applied to patients suffering from pleomorphic adenomas (smokers, n = 10; nonsmokers, n = 19; $P > 0.96$), adenolymphomas (smokers, n = 26; nonsmokers, n = 8; $P > 0.18$), carcinomas (smokers, n = 8; nonsmokers, n = 6; $P > 0.54$), and stones (smokers, n = 7; nonsmokers, n = 4; $P > 0.1$). Tumor volumes as determined by ultrasound-guided sizing were not found to be correlated with leptin concentrations in saliva samples (not shown).

Discussion

Several studies have reported the presence of leptin and its receptors in human salivary glands *(8, 17, 16)*. Moreover, leptin or its receptors have been observed in gastric, colorectal, and breast cancers. The hormone influences cell proliferation *(20, 24–26)* and seems to be associated with cancer risk *(27, 28)* and the progress of invasiveness in cancers *(10–13)*. One possible reason for the association of tumors with these processes could be stimulation of vascular endothelial growth factor and endothelial growth factor expression, resulting in enhanced angiogenesis *(29, 30)*. Our observation that leptin and its receptors are found in salivary gland tumors in much higher amounts than in healthy tissue seems to support the theory that leptin may be involved—in a not yet adequately clarified manner—in the process of tumorigenesis. We have been unable to determine in cell culture whether growth and differentiation of the tumors leads to overexpression of leptin or what part leptin itself contributes to the development of the tumors. To the best of our knowledge, however, this study is the first to show that leptin might serve as a diagnostically relevant marker to identify tumors.

Immunoblots showed that, particularly in salivary gland tumors, leptin seems to be present as oligomers. However, as far as we know, leptin oligomers have never been reported to be present in blood serum. The fact that leptin may be secreted in the gastrointestinal tract in the form of dimers was also reported by Cammisotto et al. *(31)*. We confirmed the presence of leptin in the form of oligimers by using 4 different antileptin antibodies. Under several denaturating conditions it was not possible to separate those complexes, a result that could argue against noncovalent or disulfidic bonds. Within the intestine, effects of leptin depend on whether the cytokine is acting on the luminal or basolateral side of the epithelia *(32)*. Whether the postulated leptin oligomers are specific for or have a specific function within the gastrointestinal tract, and whether they behave differently from the monomers, requires further elucidation.

Results of our quantitative real-time PCR studies showed that leptin is actively produced by tumor cells and not imported from the blood serum. The high content of leptin within the tumor tissue was reflected in high concentrations of leptin also seen in saliva. Leptin-mRNA levels produced by the glands, when related to ApM-1–mRNA levels, seemed to represent very well the leptin concentrations found in saliva samples across all groups. Glandular adipocytes did not seem to influence the amount of leptin in saliva.

We were able to show that the analysis of leptin concentration in whole saliva samples might be used as a diagnostic marker to identify salivary gland tumors and to differentiate between tumors and other processes such as cysts and stone-dependent inflammation that can lead to enlargement of the salivary glands. Correlating salivary leptin concentrations to patient BMI appears to supply useful information for differentiating between stones and cysts. Owing to the small number of saliva samples we examined that were from patients suffering from stones and cysts, further investigations are necessary to verify this supplemental result of our study.

It is noteworthy that various swab materials used in the collection devices influenced the results tremendously. All our samples were obtained with Salivettes equipped with polyester swabs, which seem to supply the most reliable data *(33)*. Saliva obtained selectively from affected glands may show even higher differences in leptin levels.

Because all samples for this study were obtained within the last 2 years, follow-up data are not yet sufficient for publication. At present, we do not know if relapses have occurred in participating benignoma and carcinoma patients. Owing to the small numbers of carcinoma patients in our investigations, we have not yet established meaningful correlations of lymph node status, vessel invasion, grading, or survival with salivary leptin levels or leptin staining in tissue samples.

Further analysis of salivary gland–derived peptides that seem suitable for early detection of tumors, as indicated by Pepe and coworkers *(34)*, could also lead to the identification of patterns that specifically indicate neoplasms in other organs. The determination of leptin in saliva appears to hold promise as a marker for the diagnosis of salivary gland tumors of diverse types.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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