Lack of RUNX3 regulation in human gastric cancer

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Abstract

It has been proposed that the transcription factor RUNX3 is the product of a gastric tumour suppressor gene. We examined RUNX3 expression in gastric biopsies from 105 patients with different histological presentations. Surprisingly, immunohistochemical staining detected RUNX3 protein expression only in infiltrating leukocytes but not in the gastric epithelium. Using laser capture microdissection and quantitative reverse transcription-polymerase chain reaction, we confirmed that the level of RUNX3 mRNA expression in the gastric epithelium was very low and was influenced neither by H. pylori infection nor by neoplastic transformation. Instead, RUNX3 was highly expressed in the gastric stroma and the level of expression correlated with the magnitude of H. pylori-induced gastric inflammation. The low level of RUNX3 expression in gastric epithelium and the absence of downregulation in gastric cancer do not support the hypothesis that RUNX3 functions as a gastric tumour suppressor gene.

Introduction

Gastric adenocarcinoma is the second leading cause of cancer-related death world wide and approximately 650 000 people die from this malignancy each year [1]. Two histologically distinct variants of gastric carcinoma have been described: the diffuse type and the intestinal type. Epidemiological and interventional studies in humans, as well as experiments in rodents, have strongly linked H. pylori infection to the development of both types of distal gastric cancer [2–5].

Many genes have been scrutinized in attempts to understand the molecular basis for human gastric cancer development [6]. However, in contrast to the well-defined sequence of genetic alterations that accumulate during colorectal carcinogenesis, no mutational events are consistently associated with initiation of, and progression to, gastric adenocarcinoma. Recently, the Runt domain transcription factor RUNX3 has been linked to gastric cancer development [7]. The RUNX genes form a family of three mammalian transcription factors with essential functions in developmental pathways regulating haematopoiesis [8,9], osteogenesis [10,11], neurogenesis [12,13], and thymopoiesis [14–16]. Furthermore, their involvement in oncogenesis is under thorough investigation. RUNX1, for example, is among the most common targets of chromosomal translocations in human leukaemia [17]. RUNX3 has been proposed to function as a gastric tumour suppressor gene. RUNX3−/− mice exhibit hyperplasia of the gastric epithelium, leading to death by starvation shortly after birth [7]. It has been suggested that RUNX3 expression in human gastric carcinoma is often downregulated owing to epigenetic silencing by promoter hypermethylation [7,18–20]. Moreover, enforced expression of RUNX3 inhibited gastric cancer cell growth in vitro as well as tumorigenicity and metastasis in vivo [7,21]. Together, these findings support the assumption that RUNX3 is a candidate tumour suppressor gene.

However, this concept has been challenged by Levanan and colleagues who generated RUNX3−/− mice on a different genetic background. These mice showed comparable phenotypes for thymopoiesis [14,16] and neurogenesis [12,13] but did not show early-onset hyperplasia of the gastric epithelium and survived for up to 2 years without developing gastric cancer [22]. Notably, while Li et al detected RUNX3 expression in the normal gastric epithelium by X-gal staining in heterozygous RUNX3LacZ/+ mice as well as by in situ hybridization [7], Levanon et al, using the same techniques, did not [22–24].

This was supported by Carvalho et al, who also reported a lack of RUNX3 expression in human non-neoplastic epithelium using immunohistochemistry [25]. Furthermore, using fluorescence in situ hybridization to evaluate the number of RUNX3 alleles...
in samples of early-onset gastric cancer and cancer-derived cell lines, the authors found that, although loss of RUNX3 alleles occurred frequently, all cells under investigation retained at least two alleles owing to polyploidy.

Gastric cancer usually has a poor prognosis. It typically invades the muscularis propria before diagnosis and 5-year survival rates are less than 20% [6]. A prerequisite for advances in the treatment of this disease is a better understanding of its biology and behaviour. Given the above-mentioned controversy, we aimed to clarify the role of RUNX3 as a gastric tumour suppressor and to define the influence of *H. pylori* infection on its expression.

**Materials and methods**

**Patient material**

Our studies were approved by the Ethics Committee of the Medical Faculty of the Technical University of Munich. During the years 2003–2005, a total of 105 patients were examined after obtaining informed consent. Patients underwent either routine gastroscopy owing to abdominal complaints or gastrectomy owing to gastric cancer. Patient material was obtained consecutively from one ward of the 2nd department of medicine or immediately after surgery, respectively. Patients taking non-steroidal anti-inflammatory drugs or receiving anti-secretory therapy and patients with cancer receiving neoadjuvant chemotherapy were excluded from the study.

In diffuse-type gastric cancer a precise separation of cancer cells and stroma is difficult to achieve without cross-contamination. Therefore, only tumours of the intestinal type were used. Table 1 gives an overview of tumour staging and grading in the patient group. *H. pylori*-negative cancer patients were excluded to eliminate a confounding variable. Biopsy and gastrectomy samples underwent histological evaluation by two different histopathologists. The inflammatory response in the mucosa towards *H. pylori* was evaluated according to the updated Sydney classification system in regard to the degree of granulocytic infiltration (G1°, mild; G2°, moderate; G3°, severe) and lymphocytic infiltration (L1°–L3°). Patients showed different histological presentations: (N) normal stomach (n = 24, median age 52 years, 58% female, 42% male); (L0/L1) gastric inflammation with low lymphocytic infiltration (n = 10, median age 62 years, 50% female, 50% male); (L2/L3) gastric inflammation with high lymphocytic infiltration (n = 10, median age 56 years, 70% female, 30% male); (AT/IM) atrophic gastritis and/or intestinal metaplasia (n = 25, median age 64 years, 40% female, 60% male); (CA) gastric carcinoma (n = 36, median age 72 years, 44% female, 56% male). The overall median patient age was 63 years ranging from 18 to 88 years; 49% female, 51% male patients. All patients except the healthy control population were *H. pylori* positive.

**Tissue preparation and microdissection**

Under RNase-free conditions, formalin-fixed, paraffin-embedded tissue samples were sectioned at 10 μm, mounted on non-coated glass slides and processed without delay. For microdissection, slides were de-waxed in two changes of xylene, rehydrated, stained with haematoxylin-eosin (H&E) and finally immersed in 100% ethanol. From similarly treated slides, whole tissue was scraped off to extract total RNA. Microdissection was performed using the Arcturus PixCell IIe Laser Capture Microdissection System (Arcturus Bioscience, Inc., Mountain View, CA, USA). At least 1000 cells per sample were obtained.

**RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)**

Microdissected cells and scraped-off tissue were immediately immersed in 200 μl lysis buffer containing 10 mmol/l Tris/HCl (pH 8.0), 0.1 mmol/l EDTA (pH 8.0), 2% sodium dodecyl sulphate (pH 7.3), and 500 μg proteinase K (all chemicals from Applichem, Darmstadt, Germany), and incubated for 16 h at 60°C until completely lysed. RNA was then extracted by the phenol/chloroform method and precipitated with an equal volume of isopropanol, 0.1 volume of 3 mol/l sodium acetate, and 20 μg carrier glycogen (Roche, Mannheim, Germany) at −20°C. Subsequently, the RNA pellet was washed once in 70% ethanol.

| Table 1. Histopathological staging and grading of the 36 patients with gastric cancer |
|--------------------------------------|----|----|----|----|----|----|----|----|
| | N0 (n = 19) | N1 (n = 11) | N2 (n = 5) | N3 (n = 1) | M1 (n = 2) | G1 (n = 4) | G2 (n = 12) | G3 (n = 20) |
| T1 | 13 | — | — | — | — | 3 | 8 | 2 |
| | (n = 13) | (100) | (50) | (25) | (50) | (100) | (50) | (25) |
| T2 | 5 | 10 | 2 | 1 | 1 | (23) | (62) | (15) |
| | (n = 18) | (28) | (56) | (11) | (5) | (5) | (17) | (78) |
| T3 | — | 13 | 1 | — | — | — | 3 | — |
| | (n = 4) | (25) | (75) | (25) | (75) | (25) | (75) | (25) |
| T4 | 1 | — | — | — | — | (100) | — | — |
| | (n = 1) | (100) | (100) | (100) | (100) | (100) | (100) | (100) |

TNM classification of malignant tumours (UICC 6th edition, 2002). M-status at the time of surgery. If not denoted differently, numbers in parentheses are percentages of patients at the given T-stage.

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ethanol, dried, and resuspended in 20 µl of RNAse-free water. RNA samples were digested by DNase I (Invitrogen, Karlsruhe, Germany) to remove possible contamination by genomic DNA, then transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions.

Quantitative RT-PCR

Quantitative TaqMan® real-time RT-PCR was performed using the ABI PRISM 7700 sequence detection system as described previously (Applied Biosystems, Foster City, CA, USA) [26]. A mastermix was prepared on ice containing 12.5 µl Absolute™ QPCR ROX Mix (ABgene, Hamburg, Germany), 0.5 µl each of primers and fluorogenic probe, and 6 µl of H2O, in order to obtain a total volume of 25 µl when adding 5 µl of cDNA. Reaction conditions were 2 min initial incubation at 50°C, followed by 15 min at 95°C to activate the hot-start polymerase, and continued with 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 30 s. All reactions were performed in duplicate. Absolute copy numbers of RUNX1/3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained by generating standard curves for each mRNA using plasmid dilution series. RUNX1/3 mRNA copy numbers were normalized to 10^4 GAPDH copies. Primer and probe (MWG Biotec, Ebersberg, Germany) sequences were as follows: RUNX1-fw (5-CTCAGCCTCACAGTACAGGCA-3), RUNX1-rv (5-GGTATTGGTAGGACTGATCGTAGGA-3), RUNX1-probe (5-ACAAGGCAGATCCAACCATCCACCCAGGATGTAAGG-3), RUNX3-fw (5-GCCGTCTCATCCCATACCTTCTG-3), RUNX3-rv (5-TAGCTGGAGACAGTGA GGTCCTT-3), RUNX3-probe (5-AGCCTCCTGCCACCCACCCCA-3), GAPDH-fw (5-GGGAAGCTTGTCA TTCAATGGA-3), GAPDH-rv (5-CGCCACTCATCGTCCAGGAGCG-3), and GAPDH-probe (5-ATCCCATCACCTTCCAGGAGCG-3).

Statistical analysis

The Mann–Whitney rank sum test was used to compare RUNX1/3 expression in different patient groups. The significance level was set at 0.05 two sided.

Immunohistochemistry

Immunohistochemistry was performed as outlined in Carvalho et al [25]. Briefly, paraffin sections were incubated with purified anti RUNX1 and RUNX3 antibodies (1:100 and 1:1000 respectively) in phosphate buffered saline containing 0.1% Triton X-100 and 3% normal goat serum. Biotinylated secondary antibodies and Vectastain ABC complex (Vector Laboratories, Burlingame, CA, USA) were used for signal detection.

Results

RUNX3 mRNA expression in gastric biopsies does not differ between neoplastic and non-neoplastic mucosa but depends on H. pylori infection

Several studies have analysed RUNX3 mRNA expression in the gastric mucosa of patients with various clinical presentations and showed downregulation of RUNX3 expression in gastric cancer [18–20]. We used real-time quantitative RT-PCR to evaluate RUNX3 mRNA expression in human gastric specimens from 105 patients with different histopathological presentations (Figure 1A). In contrast to the previously published data, there were no significant differences in RUNX3 expression between the three groups. Importantly, RUNX3 mRNA levels were not reduced in

Figure 1. RUNX3 mRNA expression in human gastric biopsy samples from 105 patients. (A) RUNX3 expression in patients with different histological presentations: N/G, normal stomach or mucosa with gastric inflammation; AT/IM, atrophic gastritis and/or intestinal metaplasia; CA, gastric carcinoma of the intestinal type according to Lauren’s classification. RUNX3 mRNA copy numbers were normalized to 10^6 GAPDH copies. Boxes represent the 25th, 50th and 75th percentiles, whiskers the 10th and 90th percentiles, and the 5th and 95th percentiles are shown by filled circles. There were no significant differences between the three groups (Mann–Whitney rank sum test). (B) RUNX3 mRNA levels in antral biopsies from H. pylori infected and non-infected patients. Patients with severe histopathological alterations of the gastric mucosa (atrophy, intestinal metaplasia, carcinoma) were excluded from this graphic. Differences between the two groups were significant (p < 0.01; Mann–Whitney rank sum test)
gastric cancer samples. RUNX transcription factors share the highly homologous Runt domain (RD) that mediates DNA binding and interaction with the protein core binding factor β. The RD mRNA sequences of the three human RUNX genes show more than 80% homology. In contrast to the primers used in this study, the primer sequences used in the above-mentioned studies were located in the RD (or could not be matched to RUNX3 at all), which may be one explanation for the contradictory results to our examinations.

Given the well-established association of H. pylori infection and aberrant CpG methylation on the chronically inflamed gastric mucosa [27], we sought to determine a possible association of RUNX3 expression with H. pylori infection. We therefore analysed RUNX3 mRNA expression in relation to H. pylori status in the above-mentioned 44 patients who did not have severe histopathological mucosal alterations (N/G group). The presence of bacteria in the stomach was investigated by immunohistochemistry and PCR from gastric biopsies, as described previously [28,29]. Figure 1B shows that RUNX3 expression was significantly increased in the gastric mucosa of H. pylori infected patients, compared with non-infected subjects (p < 0.01).

RUNX3 protein is expressed in gastric leukocytes but not in human gastric epithelium

H. pylori infection induces infiltration of the lamina propria with leukocytes that may express RUNX3. To assess the cellular origin of RUNX3 expression in the human gastric mucosa we performed immunohistochemical RUNX3 staining on stomach sections. In the inflamed stomach, positive RUNX3 staining was seen in leukocytes but was almost completely absent in epithelial cells. Figure 2A and B shows the nuclear staining of RUNX3. In contrast, RUNX1 protein was expressed in both gastric epithelial cells as well as leukocytes (Figures 2C and D). The lack of RUNX3 staining in the non-cancerous gastric epithelium makes it unlikely that RUNX3 has a role in gastric carcinogenesis. As RT-PCR may be a more sensitive method to assess low-level RUNX3 expression, we next quantified RUNX3 mRNA expression in different compartments of microdissected gastric tissue.

RUNX3 mRNA expression in microdissected gastric epithelium is not downregulated upon neoplastic transformation

To determine whether H. pylori influences RUNX3 expression in epithelial cells, we laser-microdissected gastric tissue samples from all patients, thereby separating epithelial cells or cancer cells from the surrounding stromal tissue. RUNX3 mRNA levels could then be determined individually in those two compartments. Surprisingly, RUNX3 expression was mainly restricted to the stroma (Figure 3A), whereas epithelial RUNX3 mRNA levels were very low. In contrast, RUNX1 expression in the epithelial compartment was similar to that found in the stroma (Figure 3A).
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We subsequently analysed RUNX3 expression in relation to the histopathological appearance of the gastric mucosa (Figure 3B). Therby, we found that RUNX3 mRNA levels in the stroma strongly depended on the inflammatory score. As shown in Figure 3B, RUNX3 expression was increased in patients with medium/severe gastritis compared with patients with normal histology or mild gastritis (p < 0.001). However, analysing gastric epithelial RUNX3 expression, we found no differences between normal, metaplastic or neoplastic epithelia (Figure 3B). Furthermore, H. pylori infection was associated with increased RUNX3 mRNA levels in the stroma, reflecting the inflammatory response induced by the bacteria. However, infection did not influence epithelial RUNX3 expression (not shown). These findings suggest that RUNX3 expression in whole gastric biopsies is mainly determined by the inflammatory infiltrate and is not regulated in epithelia.

Discussion

The role of RUNX3 as a possible gastric tumour suppressor gene is controversial [7,24,25,30]. Our results do not support the hypothesis that RUNX3 acts as a gastric tumour suppressor gene, but they provide explanations for some of the discrepancies between previous studies. Immunohistochemical staining of gastric tissue samples showed that mucosal RUNX3 expression is confined to infiltrating leukocytes and cannot be detected in the epithelium. The analysis of mRNA expression by TaqMan RT-PCR is more sensitive for the detection of low-level RUNX3 expression. Our microdissection experiments revealed that differences in mucosal RUNX3 expression between healthy, inflamed, metaplastic or neoplastic gastric tissue are a consequence of differential RUNX3 expression in the stroma and correlate strongly with the extent of inflammation. In contrast, RUNX3 expression in the gastric epithelium is unchanged, regardless of the histological diagnosis. Also, H. pylori infection does not influence epithelial RUNX3 expression, but induces infiltration of the mucosa with RUNX3-positive leukocytes. Previous reports showing downregulation of mucosal RUNX3 mRNA expression in gastric cancer specimens did not perform microdissection and may therefore have been biased by the choice of inflamed mucosa as a control tissue. Given the high prevalence of H. pylori infection world wide, this may not be unlikely.

Loss of function in both alleles of a tumour suppressor gene is required to drive a neoplastic process [31]. For RUNX3, it has been postulated that epigenetic silencing by promoter hypermethylation may have a role in this process [19,32]. However, it is not clear whether or not this epigenetic event causes carcinogenesis or is just a common event in the deregulated growth of already established carcinoma [33,34]. Clearly, epithelial RUNX3 expression was not reduced in gastric tissue samples, strongly suggesting that epigenetic events do not play a major role. In addition, no RUNX3 mutations that are associated with gastric cancer have been identified so far. Somatic loss of heterozygosity is the main mechanism leading to the complete loss of function of tumour suppressor genes and subsequent malignant transformation, and RUNX3 loss of heterozygosity has been proposed to be a causative mechanism in human gastric cancer [7]. However, owing to frequent aneuploidy, tumour cells in early-onset gastric carcinoma retain at least two alleles [25], thereby making loss of heterozygosity as a causal event in gastric carcinogenesis unlikely. Thus, our results together with previous observations by other groups do not support a model in which RUNX3 is a key player in gastric carcinogenesis.

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