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Under-expression of miR-100 may be a new Carcinogenic pathway for low-grade pTa Bladder Urothelial Carcinomas

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Abstract

Objectives: The pathways involved in the carcinogenesis of bladder urothelial carcinoma have been well established and are used for the development of new diagnostic and prognostic markers for the disease. The main genetic pathway for the development of low-grade pTa urothelial carcinomas is related to FGFR3 mutation. MicroRNAs have been related to processes involved in carcinogenesis in many organs, and miR-100 was recently shown to target FGFR3 messenger RNA. Our aim was to study the profile of expression of miR-100 and FGFR3 in low-grade, pTa bladder urothelial carcinoma.

Methods and Materials: Using qRT-PCR, we studied the expression of miR-100 and FGFR3 in 30 patients who had undergone transurethral resection of low-grade, pTa bladder urothelial carcinoma.

Results and Conclusion: There was under-expression of miR-100 and over-expression of FGFR3 in 100% of the specimens. Under-expression of miR-100 might be an alternative pathway for low-grade pTa urothelial bladder carcinogenesis and the identification of this molecular alteration may constitute a new diagnostic and prognostic marker for the disease.

Keywords: Micro RNA; Bladder cancer; Carcinogenesis; Molecular pathway; Prognosis; Diagnosis; FGFR3

Introduction

Bladder cancer (BC) is the second most common malignancy of the urinary tract. Approximately 383,300 new cases are estimated for 2011 [1]. Ninety percent of BC are urothelial carcinomas (UC), previously named transitional cell carcinomas, and the majority are papillary low-grade, non-muscle invasive that recur in up to 80% of cases but rarely progress to muscle invasion [2-3]. In contrast, 10 to 20% of tumors are muscle invasive at diagnosis, and 50% of patients die from metastatic disease [4].

The molecular pathways underlying the main two distinct types of UC, low-grade, non-muscle invasive, and high-grade, muscle invasive [2,5] have been investigated to identify new potential markers for diagnosis, disease monitoring, prognosis and development of new targeted therapies [2,6,7]. The most common genetic alteration of BC associated with low-grade and stage neoplasias is an activating mutation of the fibroblast growth factor receptor 3 (FGFR3) gene [8-9], whereas mutations in the p53 and retinoblastoma (RB1) genes have as being identified as characteristic of the carcinogenesis pathway for high grade invasive disease [5,7,10,11].

The FGFR3 gene belongs to the growth factor receptor family related to the tyrosine kinase signaling pathway, which plays an important role in embryogenesis, development, angiogenesis, wound healing, tissue homeostasis and tumorigenesis, regulating cellular proliferation, migration and apoptosis [9]. Mutations are the primary phenomenon related to FGFR3 dysfunction allowing its ligand-independent operation [9,12] and the identification of FGFR3 dysfunction has been purposed as a new tumor marker [5,10,13]. Other events that may lead to the over-expression of the FGFR3 including alterations in the epigenetic control as DNA methylation, histone acetylation and abnormalities in the expression of microRNAs can also play crucial roles in the development and progression of BC [14].

MicroRNAs are members of small single-stranded regulatory RNAs (21-25 nucleotides) that can suppress the translation or promote the degradation of mRNA regulating the expression of target genes, including transcription factors, oncogenes and tumor suppressor genes. MicroRNAs have been reported to be differentially expressed in several types of cancers. Currently, there are more than 1400 miRNAs described in humans, and up to 30% of genes are thought to miRNA regulated [15]. MicroRNAs are involved in cell development, differentiation, apoptosis, tissue homeostasis and several metabolic pathways [16-19], and they have been related to carcinogenesis acting as negative regulators of genes related to cancer as typified by the effect of miR-15a and miR-16-1 on BCL2 mRNA, miR-143 and miR-let7c on RAS mRNA and miR-21 on p53 mRNA [20-23].

Recently authors have demonstrated that the FGFR3 gene is a target of micro RNA 100 (miR-100), and overactivation of FGFR3 could be involved in the development of low-grade, non-muscle invasive UC [23,24]. Our aim was to study the expression of miR-100 and the FGFR3 gene in low-grade, non-invasive UC to validate a new carcinogenesis pathway that may be involved in the development of the disease and that could potentially be used as a new biomarker.

Materials and Methods

Patients

Thirty low-grade non-invasive pTa urothelial carcinomas obtained

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from patients who underwent transurethral resection were the subject of the study. Eighty-seven percent (26/30) of patients were male; the mean age was 67.6 years old, ranging from 47 to 82. As control, we used normal bladder tissue from five patients who had undergone retropubic prostatectomy to treat benign prostatic hyperplasia. All patients provided informed consent and the study design was approved by the Institutional Board of Ethics protocol 10/176.

A subsection of the all the specimens were fixed in 10% formalin routinely processed and stained with hematoxylin and eosin for histological examination. Only urothelial low-grade (2004 WHO/ISUP) pTa (2010 AJCC/TNM) tumors were included in the study.

RNA and miRNA extraction and amplification

Part of the resected tissue was immediately frozen and stored at -80ºC. At the time of the RNA and microRNA extraction, a fragment of the frozen tissue was fixed in 10% formalin, routinely processed and embedded in paraffin and a slide was stained with hematoxylin and eosin to guarantee the presence of tumor in at least 75% of the specimen.

miR-100 expression was examined in all 30 cases; however, because of the relatively small amount of tissue available, FGFR3 messenger RNA expression was only examined in 15 cases.

Total RNA and miRNA were isolated using a RNAaqueous Kit® (Applied Biosystems, CA, USA) and a mirVana Kit® (Applied Biosystems, CA, USA), respectively, according to the manufacturer's instructions. RNA and miRNA concentration was determined by 260/280 nM absorbance using Nanodrop® ND-1000 spectrophotometer (Thermo Scientific). cDNA was generated using a High Capacity cDNA Reverse Transcription Kit® (Applied Biosystems, CA, USA) for RNA and a Taqman MicroRNA Reverse Transcription Kit® (Applied Biosystems, CA, USA) for miRNA. RNA reactions were incubated at 25°C for 10 min, followed by 37°C for 120 min and 85°C for 5 min, and the miRNA was incubated at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. The cDNA was stored at -20°C until further use.

For mRNA and miRNA amplification, a Taqman Reagent Kit® (Applied Biosystems, CA, USA) was used in the 7500 Fast Real-Time PCR System® (Applied Biosystems, CA, USA).

Expression profiles of miR-100 and FGFR3 mRNA were obtained by relative quantification determined using the $2^{-\Delta\Delta ct}$ method. Reactions were conducted in duplicate using β-2 microglobulin (B2M) as an endogenous control for mRNA analysis and RNU-43 and RNU-48 as endogenous controls for miRNA analysis.

Statistic analysis

The distribution of the expression levels of the miRNAs was skewed; therefore, the data were log transformed for analyses. Results are presented as geometric means with a 95% confidence interval (95% CI).

Results and Discussion

The data for miR-100 and FGFR3 expression are presented in Table 1 and Figure 1. All examined cases displayed under-expression of miR-100 and over-expression of FGFR3. The mean and median expression values for miR-100 and FGFR3 were 0.038 and 0.0008 (8.94E-13 – 0.44), and 1599.6 and 202.95 (3.26 – 16002.3), respectively.

Molecular pathways are directly involved in the biological behavior of BC and are able to predict disease evolution prior to its complete presentation. Our study revealed that 100% of low-grade pTa UC showed under-expression of miR-100 (30/30) and over-expression of FGFR3 (15/15), showing a strict association between two events. We

Cases	miR-100	FGFR3
1	2.1E-08	697.34
2	0.1982	274.56
3	0.0831	240.35
4	0.0008	5556.65
5	0.0006	202.95
6	0.0003	289.01
7	0.0008	313.21
8	0.0086	112.59
9	3.4E-08	16002.39
10	0.1172	3.26
11	0.44	19.67
12	0.0726	92.34
13	0.0007	11.09
14	0.0074	64.22
15	0.0011	132.33
16	8.9E-13	-
17	1.21E-12	-
18	3.14E-11	-
19	2.87E-09	-
20	6.11E-10	-
21	2.85E-08	-
22	1.18E-07	-
23	1.31E-08	-
24	2.03E-08	-
25	0.1099	-
26	0.0014	-
27	0.0030	-
28	0.0732	-
29	0.0237	-
30	0.0009	-

Table 1: miR-100 and FGFR3 expression values.

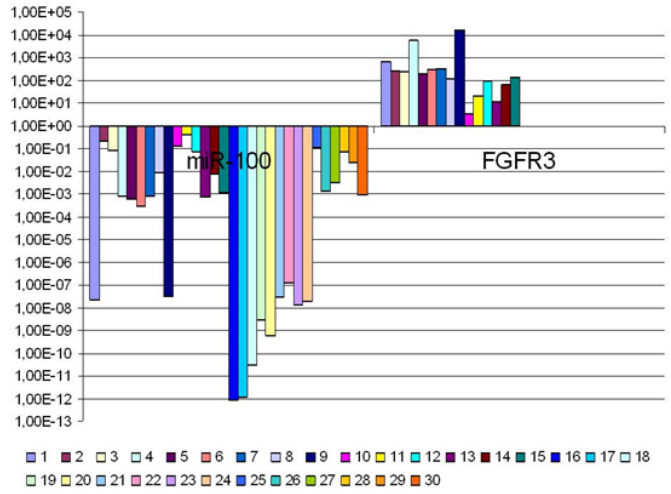

Figure 1: Expression levels of miR-100 and FGFR3. Note under-expression of all low-grade non-invasive pTa tumors and 100% of robust over-expression of FGFR3.

sought to prove that FGFR3 is a target of miR-100, and that FGFR3 over-expression is due to the under-expression of miR-100, which is a different mechanism related to BC carcinogenesis that could be used in clinical practice. Catto et al. (2009), who studied 22 cases of low-grade pTa UC, found the same result and proved FGFR3 as a target for miR-100 in vitro models.

The FGFR3 mutation in BC was initially described in 1999 by Cappellen et al., who identified mutations in 35% of the tumors they examined. FGFR3 can be abnormally activated through two mechanisms. The first mechanism is the translocation t(4;14)(p16.3; q32) that results in an increase in mRNA, as has been described in multiple myeloma [26]. The second mechanism is an anomalous over-activation of the receptor by a point mutation [27]. Currently, the FGFR3 point mutation is well established in BC carcinogenesis; it is present in more than 70% of low-grade non-invasive UC [5,9,13,23], and can be caused by tobacco smoke and contact with aromatic amines [28,29]. These mutations activate the tyrosine kinase domain, lowering the specificity for the ligand, promoting dimerization independent of the ligand or inducing autophosphorylation of the intracellular domain.

In contrast, approximately 30% of low-grade pTa UC do not have the FGFR3 mutation suggesting that other regulatory pathways may be involved in the pathogenesis of the tumor. Few studies have evaluated alternative mechanisms of FGFR3 dysfunction in BC [13,23].

It is important to clarify the mechanisms that may be involved in the dysfunction of miR-100 expression. Blick et al. (2011) recently demonstrated that hypoxia could up-regulate FGFR3 and concomitantly down-regulate miR-100 in an HIF-1a dependent manner in BC. In addition, epigenetic events such as methylation could be involved in the silencing of miR-100 [31].

We hypothesize that mechanisms affecting miR-100 expression could be the first molecular event in low-grade pTa UC development, and there is a need for further studies to confirm the precise role of miR-100 as a controller of FGFR3 in BC. If these events precede FGFR3 mutation, we propose the search for miR-100 under-expression as a new tumor diagnostic and prognostic marker.

We have demonstrated a profile of miR-100 and FGFR3 expression in low-grade pTa UC that could precede or be an alternative pathway for bladder carcinogenesis. Further confirmation of the findings presented in this study would constitute new data for the development of novel diagnostic and prognostic markers for bladder carcinogenesis.

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