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RESEARCH

Expression and Amplification of Topoisomerase-2α in Type 1 and Type 2 Papillary Renal Cell Carcinomas and Its Correlation with HER2/neu Amplification

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Abstract The current study was undertaken to investigate chromosomal and genetical aberrations leading to overexpression of Topoisomerase-2α (TOP2α) and to reveal the possible association of these aberrations with HER2/neu overexpression and gene amplification, and to search for the relationship between TOP2α and HER2/neu status with prognostical biomarkers in papillary renal cell carcinoma (RCC), a group of tumors with diverse molecular, chromosomal and clinical features. Archival cases of papillary RCC obtained from Departments of Pathology of Pamukkale, Ege and Dokuz Eylul Universities were studied in two groups (type 1 and type 2) each containing 20 cases. The level of TOP2α and HER2/neu expression by tumor cells were determined immunohistochemically. A multicolor FISH probe was used to define both amplification of HER2/neu and TOP2α genes, and polysomy 17. The ratio of cells expressing TOP2α in type 1 and type 2 papillary RCC were 24.29% and 6.89%, respectively. The difference was statistically significant comparing the average or median values of groups separately (p=0.002). The expression levels of TOP2α and HER2/neu were also correlated. TOP2α and HER2/neu were co-amplified in both groups. Immunohistochemical expression was not observed in 15 of 23 cases with HER2/neu amplification. The most frequent finding detected by FISH method was polysomy of chromosome 17. We had contradictory results compared with the findings reported in the limited numbers of literature. It shows us that papillary RCC constitute a heterogenous group of tumors with various cytogenetic features and morphological classification of these tumors may not be compatible with their molecular characteristics.

Keywords Papillary renal cell carcinoma · Topoisomerase-2α · HER2/neu · FISH · Immunohistochemistry

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Introduction

Renal cell carcinomas (RCC), the most common malignancy of kidney, show morphological and genetical diversity. RCCs have various subtypes as clear cell, papillary, chromophobe, collecting ductus and unclassified, and 10–15% of them are papillary type [1]. Papillary RCC were classified into two subtypes by Delahunt and Eble in 1997 and this classification was accepted by WHO in 2004 [1, 2]

Type 1 tumors are composed of thin papillae covered by cuboidal cells with uniform nuclei and basophilic cytoplasm. The type 2 tumors are characterized by the presence of large cells with eosinophilic cytoplasm and pseudostratification. However, morphological features of both types may be seen in the same tumor and type 2 tumors are additionally divided into two subtypes according to their nuclear grades by some authors [1, 3, 4]. Few studies demonstrated that those two subtypes of type 2 papillary RCC have also different clinical behaviour [5, 6]. After then, cytogenetical and biological differences between two types of papillary RCCs have been searched and a mass of scientific data have been provided. Trisomy or tetrasomy 7, trisomy 17 and loss of chromosome Y are the commonest karyotypic changes in papillary RCC [1, 4]. Comparative studies show more gains of chromosomes 7p and 17p in type 1 papillary RCC than type 2 tumors. Whereas different examples of chromosomal gains and losses are observed in type 2 papillary RCC [7]. It has been proposed that different molecular pathways are effective in growth of type 1 and type 2 papillary RCC and the relationship between 17q and papillary RCC subtypes may have an independent effect on prognosis. Trisomy 17, isochromosome 17q or 17q21-qter dublication, the most common chromosomal abnormalities seen in papillary RCC, support the view that expression or allelic dosage increase of a gene at 17q play an important role in growth of these tumors [8–12]. A few studies demonstrated topoisomerase IIα (TOP2α) overexpression by immunohistochemistry and claimed that this overexpression was correlated with agressive clinical behaviour [13].

Topoisomerase II cuts DNA helix during DNA replication making a double-strand break at the same time and relaxing both of the strands. Besides, topoisomeras II has been shown to effect chromosome condensation and separate of sister chromatids at mitosis [14].

TOP2α gene is located in HER2/neu amplicon at the chromosome 17q12-q21. The early studies defined TOP2α gene amplification and deletion in 80–90% of patients with breast cancer with HER2/neu amplification, and it was predicted that TOP2α gene aberrations might be seen very rarely in tumors without HER2/neu amplification [15]. However, amplification of TOP2α was found to be more frequent than HER2/neu in studies on different types of human cancers [16, 17]. More detailed studies focusing on this subject demonstrated that 17q12-q21 region has at least two amplicons including various numbers of genes [15].

The current study was undertaken to investigate chromosomal and genetical aberrations leading to overexpression of TOP2α and to reveal the possible association of these aberrations with HER2/neu overexpression and gene amplification, and to search for the relationship between TOP2α and HER2/neu status with prognostical biomarkers such as nuclear grade and tumor stage in papillary RCC.

Materials and Methods

Archival cases of papillary RCC obtained from Departments of Pathology of Pamukkale, Ege and Dokuz Eylul Universities were studied in two groups (type 1 and type 2) each containing 20 cases. The slides of each case were reclassified by three pathologists and some histopathological parameters effective on prognosis such as nuclear grade, tumor stage, lymphovascular and renal vein invasion were evaluated. After then, formalin fixed tissue samples representing the most informative areas of tumors of each case were stained by anti- TOP2α (clon: Ki-S1) and anti-HER2/neu (clon: CB11). The level of protein expression was determined as the percentage of positive tumor cells and stated as indicated in Table 1. At the same time, consecutive slides were stained by Fluorescence in situ Hybridization (FISH).

The FDA-approved FISH assay, PathVysion (Vysis, Abbot Laboratories, IL, USA), was used for the identification and quantification of HER2/neu and TOP2α genes and chromosome 17 alteration on formalin-fixed and paraffin-embedded (FFPE) sections fixed on slides according to manufacturer's recommendations. Briefly, the sections were deparaffinized in xylene and subjected to pretreatment including protease digestion for 20 min at 37°C following fixation with 10% buffered formalin and treatment with denaturation solution supplied in the kit. The pre-warmed probe mixture containing the HER2/neu, TOP2α DNA probes and the CEP 17 DNA probe was applied to the slides. After overnight (12–16 h) hybridization at 37°C, the slides were washed with post-hybridization wash buffer and counterstained with 0.2 μM 4,6-diamino-2-phenylindole

Table 1 The scoring criteria of immunoreactivity for TOP2α and HER2/neu

TOP2α immunoreactivity
♦ Negative (<0,1% positive tumor cells)
♦ Focally positive (0,1–10% positive tumor cells)
♦ Positive (>10% positive tumor cells)
Her2/neu immunoreactivity
♦ Score 0 (0–10% of tumor cells immunostained)
♦ Score 1 (>10% of tumor cells with weak partially membranous immunostaining)
♦ Score 2 (>10% of tumor cells with weak or moderately complete membranous immunostaining)
♦ Score 3 (>10% of tumor cells with severe complete membranous immunostaining)

(DAPI). Slides were preserved at −20°C in the dark before signal enumeration. A minimum of 60 non-overlapped tumor cell nuclei were analyzed using a Ziess fluorescence microscope (Ziess, Germany) and MetaSystems Isis V5.3 analyser (MetaSystems, Germany) equipped with DAPI, red-green-blue (RGB) bandpass filters.

Two signals for each locus (red for HER2/neu, green for TOP2α and blue for centromere 17) were expected in normal cells. Amplification of the HER2/neu and TOP2α were evaluated at a ratio of 2.0 or greater per centromeric 17 signals. The relative increase in HER2/neu and TOP2α copy number was determined when there were more HER2/neu and TOP2α signals than CEP 17 signals. Polysomy 17 was defined as a mean CEP 17 and locus specific signal count of 3.0 or higher per cell.

The results of immunohistochemical investigation and FISH were analyzed and correlated with prognostic parameters.

Results

The rate of TOP2α overexpression and the FISH findings of each case correlated with HER2/neu status and pathological tumor stage as shown in Table 2. Microscopic views and TOP2α overexpression of type 1 and 2 papillary renal cell carcinomas are shown in Figs. 1 and 2, respectively.

TOP2α overexpression was higher in type 1 papillary RCC and the difference was found to be statistically significant (p=0.002). The mean and median values of TOP2α overexpression are shown in Table 3. Only eight of 23 cases with HER2/neu amplification showed TOP2α overexpression. The remaining 15 cases had no TOP2α overexpression immunohistochemically.

FISH analysis showed no significant difference between type 1 and type 2 papillary tumor groups either with polysomy or amplification (Figs. 3 and 4). Polysomy 17 was the most frequent abnormality in both groups. The polysomy rate between the tumor types was insignificant, 15 (75%) and 17 (85%) in type 1 and type 2 cases, respectively. However, amplification was together with polysomy in most of the cases, 10 (66%) type 1 and 9 (52%) type 2. Polysomy 17 was the single chromosomal abnormality in only 5 (25%) type 1 and 8 (40%) in type 2 samples.

TOP2α and HER2/neu gene copy number was equal in all of the samples analyzed by FISH, showing that TOP2α

Table 2 The rate of TOP2α overexpression, Her2 status, pathological tumor stage and the FISH findings of each case

	Type 1							Type 2						
No	Stage	NG	TOP2α (%)	Her2 score	FISH results			Stage	NG	TOP2α (%)	Her2 score	FISH results		
					Normal	Polysomy	Amplification					Normal	Polysomy	Amplification
1	1b	1	39,37	0			+	3a	3	13,45	0		+	
2	1a	1	27,33	0			+	3b	3	8,00	0		+	+
3	1b	1	0,12	0		+	+	3b	2	8,38	0		+	+
4	1b	1	16,29	0	+			1a	2	3,92	0		+	
5	1a	1	41,78	0			+	1b	3	8,44	0		+	+
6	3a	1	4,11	1		+	+	1b	2	12,90	0		+	
7	2	2	6,00	0		+	+	1b	2	6,30	0		+	
8	1b	2	3,15	1		+	+	1a	2	2,77	1		+	+
9	3b	2	2,74	0		+	+	1a	3	1,20	0	+		
10	1b	2	95,01	1		+	+	2	3	0,57	0		+	
11	1a	2	32,55	1		+	+	1b	3	7,00	0	+		
12	1b	1	19,72	1		+		1a	2	4,49	1		+	+
13	1b	2	22,11	1		+	+	1a	3	2,10	0		+	
14	1b	2	17,50	0		+		1b	3	1,10	0		+	+
15	2	2	0,93	0		+		1a	2	1,53	1		+	
16	1a	2	9,76	1		+	+	1b	2	36,98	0		+	+
17	1b	2	45,00	0		+		1a	3	7,10	0	+		
18	1a	2	16,66	1		+		2	3	4,30	0		+	
19	1a	2	43,51	0		+	+	1b	3	2,43	0		+	+
20	1a	2	52,23	0			+	3	3	2,48	0		+	+

NG Nuclear grade

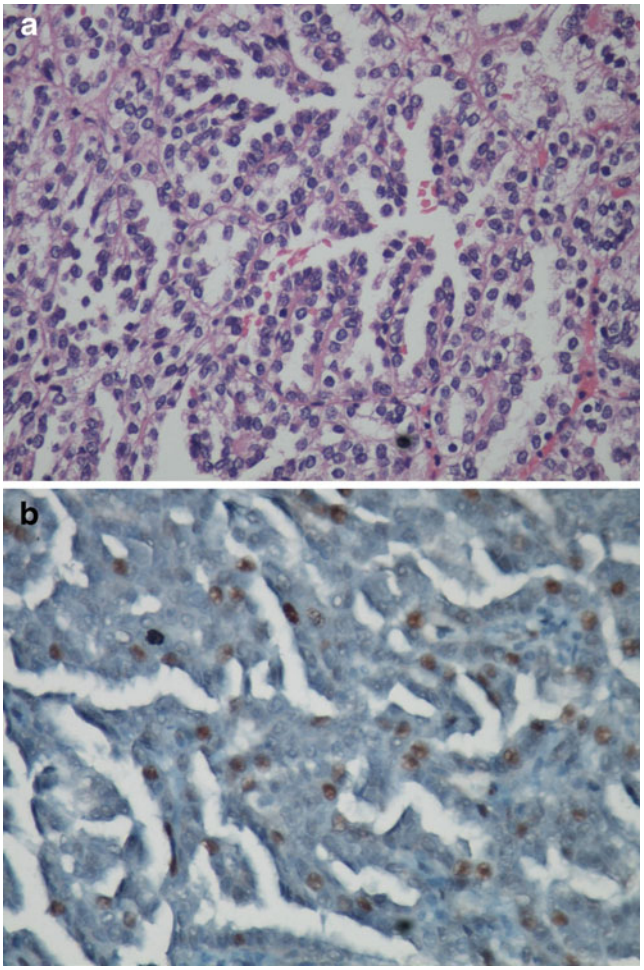

Fig. 1 **a** Microscopic view and **b** TOP2α overexpression of type 1 papillary renal cell carcinoma

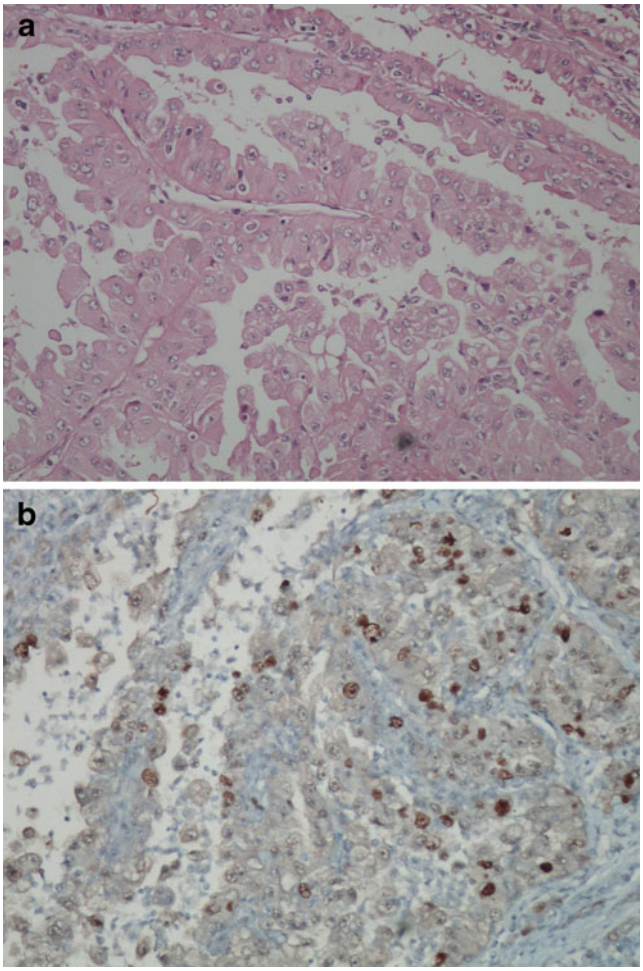

Fig. 2 **a** Microscopic view and **b** TOP2α overexpression of type 2 papillary renal cell carcinoma

and HER2/neu amplifications occurred synchronously in both groups. Increased gene copy number of TOP2α and HER2/neu was detected in 23 (57.5%) cases, 4 (17.4%) of them being as a single anomaly while others together with polysomy17. Most of the cases having unaccompanied copy number increase were type 1 and showed increased TOP2α expression while "0" HER2/neu score immunhistochemically. Three of four cases with normal chromosomal constitution were type 2 with nuclear grade 3 and also HER2/neu score was 0 and TOP2α expression was low.

Discussion

The most impressive result of the study was the higher expression of TOP2α in type 1 papillary RCCs immunohistochemically. Whereas, in some of reports, it has been emphasized that TOP2α expression was more common in high grade papillary RCCs showing aggressive tumor behavior. Dekel et al. have found a positive correlation between TOP2α index and aggressive behavior of the tumor [13]. Yang et al. used comparative genomic microarray analysis to reveal gene expression profile of 34 tumors and they divided papillary RCCs to two subgroups different form the morphological classification. They concluded that TOP2α expression was higher in the second group containing high grade type 2 papillary RCCs [18]. In

Table 3 Mean and median values of TOP2α expression levels in both groups

Tumor type	Mean (IR)	Median (SD)
Type 1	18.61 (34–44)	24.29 (22.39)
Type 2	4.49 (6–14)	6.89 (7.84)

SD Standart deviation

IR Interquartile range

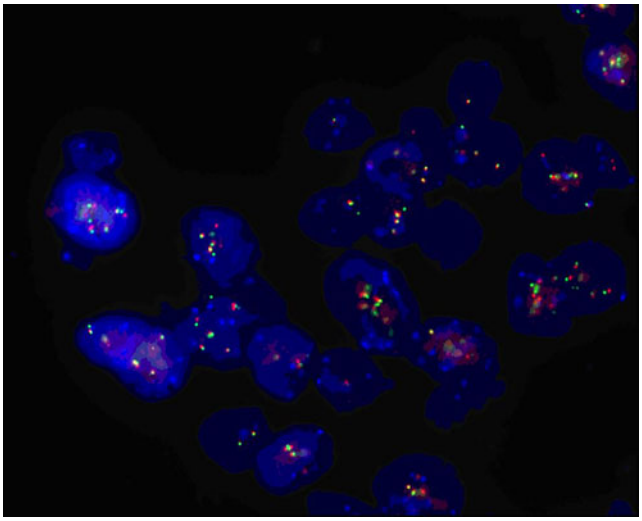

Fig. 3 FISH images showing normal, polysomic and increased gene copy number signals in type 1 papillary RCC

our study, the mean TOP2α index was 24.29% in type I and 6.89% in type 2 tumors. Although the TOP2α expression was lower in type 2 tumors by analysis using mean and median values, case based evaluation revealed that this expression was not homogenous. The TOP2α indices of cases in type I tumors were between 0.12 and 95.01% and were 0.57–36.98% in type 2 tumors. Although the mean TOP2α index was found to be higher in type 1 tumors, 6 of 20 cases in type 2 tumors showed higher expression levels than the type 1 tumors. TOP2α expression was also not correlated with nuclear grade and pathological stage.

Polysomy 17 was the most frequent abnormality in both type of tumors. Two loci at 17q12-q21 region were signed

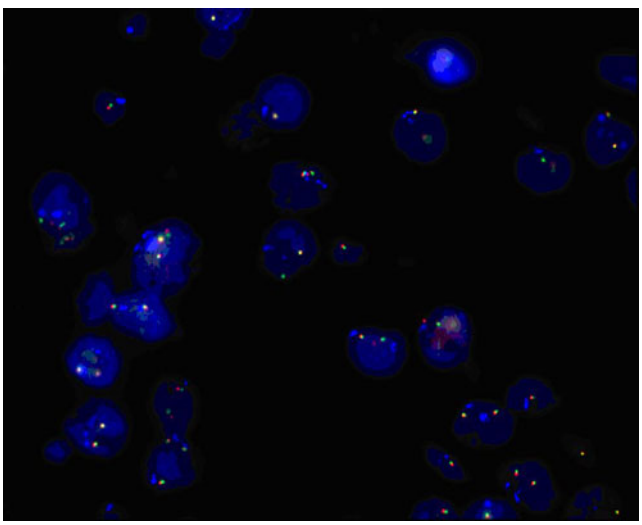

Fig. 4 FISH images showing normal, polysomic and increased gene copy number signals in type 2 papillary RCC

with FISH probes as well as centromere of chromosome 17. However, some times centromeric and locus specific signals may not change synchronously and act independently. This was explained by one or more translocations causing abnormal karyotypes [19]

There are two commonly used methods for the assessment of HER2/neu status in solid tumors: FISH and IHC. FISH was used for determining the HER2/neu gene levels while HER2/neu receptor levels on the surface of tumour cells were determined by IHC. The high level of expression observed by IHC does not always result in gene amplification, and a number of studies conducted in different solid tumors reported that tumors with amplification of HER2/neu gene were not associated with high expression of HER2/neu protein [20–22].

It was found that the increase of HER2/neu and TOP2α gene copies in the tumor cells was independent of the increased chromosome 17 copy number in this cohort. The amplification or increased copy number of HER2/neu and TOP2α gene also did not correlated with high expression of the proteins. It was reported that HER2/neu and TOP2α were not present in the same amplicon although the gene TOP2α is located in the proximal region of HER2/neu gene in chromosome 17 [23]. Therefore, the number of HER2/neu and TOP2α gene copies may be same or different in same tumor [24–26].

Immunohistochemistry is a sensitive and versatile method for the detection of specific molecules, mainly proteins, in tissue preparations or in isolated cells. Several factors could contribute to the false-negative immunohistochemical results in tumors with TOP2α amplification. One obvious explanation is the loss of antigenicity during tissue fixation or tissue processing. Bhargava et al. studied HER2/neu and TOP2α amplification and overexpression in 113 invasive breast carcinomas and found that 6 of 7 tumors showed TOP2α gene amplification without protein overexpression. Another possible explanation for observed differences in the TOP2α status might be an important role of TOP2α in DNA replication and mitotic events [26]. TOP2α is regarded as the important marker of cell proliferation because of its vital functions in cell physiology. Therefore, TOP2α protein level does not always correlate with TOP2α amplification. Expression of the TOP2α gene may also regulate with both p53-dependent and –independent mechanisms. It was found that the transcription of TOP2α promoter was decreased 15-fold by wild-type p53 in murine cells [27] and the expression of the gene is positively regulated by the binding of the nuclear factor Y transcription factor to four of five inverted CCAAT boxes located in its promoter [28].

It is well known that both genetic and epigenetic changes may lead to differences in transcriptional regulation of a gene and underlie the activation of abnormal/alternative cell

signaling pathways in human cancers. The transcriptional down-regulation or the presence of non-stabile mRNA may result in genetic variations such as mutations and polymorphisms. Changes in the epigenome by different mechanisms are also play a major role in the development and progression of human cancers. One of the important epigenetic mechanisms is non-coding RNAs identified as regulators of transcription. Non-coding RNAs act by binding to and regulating the activity of transcription factors or through the recruitment of histone-modifying enzymes [29, 30].

In this study, although we did not evaluate the clinical behaviour and prognosis of the tumors studied, it was clearly observed that TOP2α and HER2 status of renal papillary tumors does not correlate with morphological classification. Type1 and type 2 papillary renal cell carcinomas show heterogeneity by means of TOP2α and HER2 protein expression. Therefore, if it is reliable to say that there is a positive correlation between TOP2α index and aggressive behavior of the tumor according to data shown by previous investigators, the widely used morphological classification of renal papillary tumors as type 1 and type 2 is not satisfactory. A different typing system regarding molecular mechanisms responsible of aggressive behaviour of the tumors may be needed.

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