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ORIGINAL ARTICLE

Thymidylate synthetase allelic imbalance in clear cell renal carcinoma

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Abstract
Purpose To investigate the allelic status of the thymidylate synthetase (TYMS) gene, located at chromosome band 18p11.32, in renal cell carcinoma (RCC). TYMS is a key target of the 5-fluorouracil (5-FU)-based class of drugs, frequently considered in combination therapies in advanced RCC. TYMS variants, such as the TYMS polymorphic 5′-untranslated region variable number tandem repeat sequence (VNTR), are under investigation to guide 5-FU treatment. Yet, no information is available with regard to changes in TYMS allele frequencies in RCC malignances.
Methods Blood and matched tumor samples were collected from 41 histological proven clear cell RCC affected patients (30 males, 11 females.). TYMS VNTR genotype was first determined in blood to identify heterozygotes employing PCR techniques. To evaluate for allelic imbalance, fragment analysis was performed both in blood and matched tumor DNA of the heterozygote patients. Microsatellite analysis, employing the markers D18S59 and D18S476 mapping, respectively, at the TYMS locus (18p11.32) and 1.5 Mb downstream of the TYMS gene sequence (18p11.31), was performed to confirm TYMS allelic imbalance in tumors.
Results Germ-line TYMS VNTR distribution was: 2R/2R (19.5%), TYMS 2R/3R (36.6%) and TYMS 3R/3R (43.9%). Allelic imbalance for the TYMS tandem repeat region was detected in 26.6% of the heterozygote patients. Microsatellite analysis confirmed the allelic imbalance detected by TYMS VNTR analysis and revealed that the overall frequence of allelic imbalance of chromosome band 18p11.32 was 35%, while the overall allelic imbalance of chromosome band 18p11.31 was 28%.
Conclusions By focusing on the TYMS polymorphic variants in renal cancer, we here provide evidence, to our knowledge, for the first time showing loss of 18p11.32 and 18p11.31 in renal cell carcinomas. As allelic imbalances involving TYMS locus may be an important variable affecting 5-FU responsiveness, this study may contribute to explain different responses of advanced RCC in combined chemotherapeutic regimens incorporating fluoropyridines.

Keywords Thymidylate synthetase · Renal cell carcinoma · Allelic imbalance · Microsatellite analysis

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Introduction

Renal cell cancer (RCC) accounts for approximately 3% of human malignancies and its incidence appears to be rising [1]. The prognosis of RCC is mainly related to the tumor

stage (5-year survival is >90% in stage I, but <20–30% in stage IV). Unfortunately, many RCC masses remain asymptomatic and non-palpable until they are advanced and are refractory to most chemotherapeutic agents especially in their advanced stage.

A meta-analysis of 83 phase II clinical trials conducted between 1983 and 1993 in a total of 4,093 patients with advanced RCC treated with different chemotherapeutic regimens, reported a mean overall response rate of 6% [1, 2]. Other drugs used after 1993, such as platinum salts, paclitaxel [3], irinotecan [4], methotrexate-human-serum albumin [5], gemcitabine [6] and pemetrexed [7] have not shown appreciable improvement. Also, interferon-alpha (IFN-α) and interleukin-2 (IL-2), initially introduced as therapeutic agents for the treatment of metastatic RCC, have limited efficacy (response rates ranging from 11 to 17%) as well as substantial toxicity [8, 9]. On the other hand, even though controversy exists concerning the role of immunochemotherapy in the treatment of metastatic RCC, the combination of 5-fluorouracil (5-FU) with IFN-α and/or IL2 has been reported to result in improved outcome (response rate of 18–39%) as compared to immunotherapy alone [10]. 5-FU alone has a response rate of 10%, and when used in combination with gemcitabine results in improved anticancer activity (17%) [11]. This, together with the realistic probability that 5-FU will continue to be incorporated in present and future drug strategies in RCC, raises the question of whether 5-FU therapy may be better tailored in RCC patients. Although RCC chemoresistance has been correlated to the nature and presence of multi-drug resistance proteins, there is to date no information with regard to specific 5-FU pharmacogenomic determinants in RCC.

Thymidylate synthetase (TYMS), critical in DNA synthesis, is the primary target of 5-FU chemotherapy. 5-FU is, upon entry into the cell, converted by a series of enzymatic reactions to its active metabolite fluoro-deoxyuridine-monophosphate (FdUMP). FdUMP inhibits TYMS by forming a stable ternary complex with TYMS bound to its cosubstrate (5,10 methylenetetrahydrofolate) [12]. 5-FU efficacy is, therefore, directly correlated with TYMS expression levels, with low levels generally thought to predict 5-FU responsiveness, whereas high levels provide resistance and/or poor prognosis [13]. Studies employing primary cultured RCC cells have, in fact, shown that TYMS activity is positively correlated with cell sensitivity to 5-FU and that TYMS over-expression correlates with the malignant phenotype of renal cancer [14]. TYMS mRNA is up-regulated in RCC masses compared to normal tissue, and TYMS activity increases with increasing stage and grade of RCC [15]. RCC patients who undergo radical nephrectomy with low intratumoral TYMS activity have a longer disease-specific survival as compared with those with high activity in the 5-year postoperative follow-up, although multivariate analysis showed that TYMS activity was not an independent prognostic factor [14].

Given that a major mechanism of resistance to 5-FU is overexpression of TYMS, understanding the mechanisms contributing to TYMS overexpression in RCC is an essential step towards predicting or overcoming 5-FU resistance. The causes of variability in intratumoral TYMS expression are multifactorial and still unclear; however, several polymorphisms in the TYMS gene have, along with genomic aberrations in cancer, been suggested to have a significant impact. Among these, the most studied is the unique variable number tandem repeat sequence (VNTR) found in the 5′-untranslated region (UTR), immediately upstream the ATG initiation codon, that is polymorphic, containing either 2 (2R) or 3 (3R) 28-base pair repeats. Repeated sequences in TYMS affect TYMS gene expression by forming secondary structures in the 5′-terminal domain of the TYMS mRNA, with the 3R sequence having greater translational efficiency than the 2R sequence [13, 15]. However, to date, despite ongoing intensive research efforts in TYMS pharmacogenetics in a wide variety of cancer types, the role of TYMS polymorphisms or chromosomal aberrations involving the TYMS locus (chromosome band 18p11.32) in RCC have not been investigated.

We here surveyed the TYMS tandem repeat sequence in both germ-line and in matched tumor DNA samples of patients affected by RCC, along with the incidence of 18p11.32 and 18p11.31 band loss in the tumor samples, and found evidence, to our knowledge for the first time in RCC, of allelic imbalance of the short arm of chromosome 18.

Patients and methods

Patients

The study population consisted of 41 fully informed RCC patients enrolled to the Urology Surgery and Medical Oncology team in Padua (Italy) between 2004 and 2006. Patients underwent surgical treatment, and tumor tissue, together with the matched peripheral blood mononuclear cell (PBMC) samples, were used for genotype analysis. All procedures were conducted according to current Italian rules.

DNA extraction

Genomic DNA was extracted from blood samples by QiaAmp DNA Mini Kit (Qiagen, Valencia California, USA) according to manufacturer's instructions. A standard phenol–chloroform protocol was employed to perform

DNA extraction from frozen surgical specimens with a proportion of cancer cells ranging from 60 to 80%.

TYMS polymerase chain reaction

Quantitative fluorescent polymerase chain reaction (QF-PCR) specific for the TYMS tandem repeat region was conducted for all the blood and tumor-derived DNA samples. In a total volume of 50 µl, containing 100 ng of genomic DNA, 0.2 mM dNTPs, 1.25 mM $MgCl_2$, 2.5 U of Taq DNA Polymerase and 5 µl of reaction buffer 10× (all purchased from GeneCraft, Lüdinghausen, Germany) were added 22.5 µmol of forward primer, 2.5 µmol of 5′-6-FAM labeled forward primer and 25 µmol of reverse primer (MWG-Biotech AG, Ebersberg, Germany) (see Table 1). Thermal cycling consisted in an initial denaturing step of 2 min at 94°C followed by 35 cycles consisting in 30 s at 94°C, 1 min at 57°C and 1 min at 72°C. Samples displaying homozygote genotype for the triple tandem repeat variant (3R/3R) resulted in a single PCR product of 248 bp, homozygotes for the double tandem repeat variant (2R/2R) in a single product of 220 bp, while heterozygotes (2R/3R) gave both 220 and 248 bp products.

Microsatellite analysis

D18S59 (18p11.32) and D18S476 (18p11.31) microsatellite regions (respectively mapping around 11 kb upstream and 1.5 Mb downstream the TYMS gene) were amplified in a multiplex QF-PCR for all the tumor DNA samples. To 100 ng of genomic DNA were added: 0.2 mM dNTPs, 1.25 mM $MgCl_2$, 2.5 U of Taq DNA Polymerase, 5 µl of reaction buffer 10× (all purchased from GeneCraft, Lüdinghausen, Germany) 22.5 µmol of each forward primer, 2.5 µmol of 5′-6-FAM and 5′-HEX labeled forward primer (respectively for D18S476 and D18S59 microsatellite regions) and 25 µmol of each reverse primer (MWG-Biotech AG Ebersberg, Germany) (see Table 1). Thermal cycling consisted in an initial denaturing step of 2 min at 94°C followed by 30 cycles consisting in 45 s at 94°C, 45 s at 59°C and 1 min at 72°C, a final extension step of 30 min at 72°C was added to make sure that all the PCR products were completed.

Fragment analysis

A total of 1 µl of PCR products and 0.2 µl of Gene-Scan 500 LIZ electrophoresis standard (Applied Biosystems) were diluted in 8 µl of Hi-Di formammide (Applied Biosystems) and electrophoresed by ABIprism 3100 Avant genetic analyzer (Applied Biosystems). Fragment analysis was performed by GeneMapper version 3.5 (Applied Biosystems). TYMS heterozygotes electrophoresis peak ratios between 3R allele area and 2R allele area were evaluated and allelic imbalance in tumor DNA samples was attributed when the 2R/3R ratio diverged by at least of 50% when compared with the matched somatic sample [16]. Microsatellite peak analysis was performed calculating ratios between two alleles, allelic imbalance were attributed when the allele areas diverged at least by 50%.

Statistical analysis

The Kyplot v2.0 beta 15 software package [17] was used for data analysis, as well as some web pages that allow freely accessible online statistical software resources. RCC population was tested for Hardy–Weinberg equilibrium by using chi-square (χ^2) test.

Results

Forty-one histological proven clear cell RCC affected patients (30 males, 11 females mean age 61 years ± 10.4 SD) were enrolled in the study.

Germ-line TYMS genotype distribution was first evaluated to identify heterozygote samples. TYMS VNTR distribution was: 2R/2R ($n = 8$, 19.5%), TYMS 2R/3R ($n = 15$, 36.6%) and TYMS 3R/3R ($n = 18$, 43.9%) (Table 2). The observed allele frequencies were: 0.38 for the TS 2R allele and 0.62 for the 3R allele. No significant deviation from Hardy–Weinberg equilibrium was detected for germ-line genotype frequencies (χ^2 test, $P > 0.05$) indicating that the polymorphism distribution is casual in the population examined. To evaluate allelic imbalance in 2R/3R samples, fragment analysis was performed in matched tumor DNA of the 15 patients displaying heterozygote genotype.

Table 1 Primer sequences

Primer	Sequence
TS-Fw	5′-GTGGCTCCTGCGTTTCCCCC-3′
TS-Rv	5′-CCAAGCTTCGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′
D18S476-Fw	5′-ATAATCAAATCATGCCTTGGCC-3′
D18S476-Rv	5′-CCAGTATGATGGTGAAATCCTGG-3′
D18S59-Fw	5′-ATCAGTCAGCCAACCAATATCTTTT-3′
D18S59-Rv	5′-AACGACCCTCCCCTGAACTC-3′

Table 2 TYMS genotypes, tumor VNTR and microsatellite status

TYMS (VNTR) Genotypes	Germline	Tumors		
	No. patients	No. tumors[a]	D18S59 imbalance (no.)	D18S476 imbalance (no.)
2R/2R	8 (19.5%)		2	0
3R/3R	18 (43.9%)		4	4
2R/3R	15 (36.6%)	11	6	3
2R-loss/3R		1	1	1
2R/3R-loss		3	3	2[b]
Non-informative			7/41	16/41
Total number	41	15	12/34	7/25

[a] Tumors displaying TYMS VNTR imbalance employing QF-PCR (see "Methods")

[b] One TYMS imbalanced sample was homozygote for the marker D18S476

Electrophoresis peak area were quantified and 2R/3R ratio was calculated in both germ-line and tumor samples: allelic imbalance for the TYMS tandem repeat region was detected in 4 out of 15 TYMS heterozygotes samples (26.6%). Three samples (20%) displayed a tumoral 2R/3R-loss genotype and one (6.6%) displayed a 2R-loss/3R genotype (Table 2). Present data were obtained considering that partial amplification of lost alleles can result from DNA extracted from stromal, circulating and not completely transformed cells present in tumor specimens. To prevent misclassifications, and to confirm TYMS promoter region imbalance, microsatellite analysis was performed for the markers D18S59 and D18S476 mapping in the same chromosomic band and 1.5 Mb downstream of the TYMS gene sequence, respectively. Allelic imbalance revealed by TYMS VNTR analysis was confirmed by microsatellite analysis.

Finally, to better understand the status of chromosome 18 short arm in RCC pathology, D18S59 and D18S476 analysis was also performed for all the tumor samples enrolled in this study. The evaluation of the percentage of allelic imbalance did not consider homozygote samples because uninformative ($n = 7$ for D18S59 and $n = 16$ for D18S476) (Table 2). The overall allelic imbalance of chromosome band 18p11.32 was 35% (12 out of 34 heterozygote samples), while the overall allelic imbalance of chromosome band 18p11.31 was 28% (7 out of 25 heterozygote samples). All samples that displayed imbalances in the region D18S476 displayed imbalances also in the region D18S59. On the other hand 7% of samples displayed a specific allelic imbalance of the region D18S59.

Discussion

We here genotyped a series of renal cancer patients for the VNTR polymorphism located in the 5′-untranslated enhancer region of the TYMS gene and found evidence of allelic imbalance (loss of heterozygosity) in 35% of RCCs analyzed. TYMS has been localized to the telomeric region of the short arm of chromosome 18 at chromosome band 18p11.32 and, to better understand the cytogenetic status of the chromosome 18 short arm, we also investigated the allele instability of the region 18p11.31 by analyzing a marker mapping approximately 1.5 Mb downstream from the TYMS gene. Intriguingly, we observed a lower percentage of allelic loss (28%) compared with the TYMS chromosomal band. In particular, all samples displaying chromosomal imbalance in the region 18p11.31 displayed allelic imbalance also in the telomeric region 18p11.32, whereas 7% of the samples analyzed showed a specific imbalance. Even if further studies are needed, this discrepancy may be due to the presence of a fragile DNA site mapping between D18S476 and D18S59 microsatellite markers. Fragile sites, known to be tandem repeated minisatellite sequences containing high adenine and thymine percentage, are frequently associated with the presence of hotspots for chromosomal translocation, amplification, deletion and integration of exogenous DNA. Commonly, fragile sites are destabilized if exposed to replication stress and are found at the breakpoint of chromosome rearrangements, including deletions, frequently observed in tumors [18, 19].

Microsatellite analysis in conventional RCCs has shown frequent alterations on chromosomes 6q, 8p, 9, 11q, 14q, 17p, 18q, and 19p, but not on 18p [20–22]. Rather, in contrast to 18q, little is known about allelic loss or imbalance on the short arm of chromosome 18 in tumors, although a significant incidence of 18p loss (specially 18p11.32) has been found in colon, lung, brain, breast and gastric cancers [16, 23, 24]. Among these, Uchida [25] described an incidence of 77% of TYMS loss of heterozygosity (LOH) among 2R/3R VNTR heterozygote colon cancer patients who had been treated with the fluoropyrimidine-based chemotherapy. Although the 3R allele was, as expected, found to be associated with higher TYMS

mRNA levels in the tumors [13], colon cancer patients with LOH (either 2R/loss or 3R/loss) had improved survival compared with all other possible genotypes [25]. In a more recent study employing artificially constructed syngeneic cell lines mimicking the TYMS genotype in tumors, the TYMS+/− cell line (a model for a single-copy TYMS due to LOH) was found hypersensitive to 5-FU and 5-fluoro-2′-deoxyuridine (FUDR), while the TYMS-overexpressing clones (a model for tumors with increased TYMS copy number) were 5-FU- and FUDR-resistant, as compared with the parental line [26]. Moreover, in a genome-wide screening of loci associated with resistance to 5-FU-based drugs in a wide variety of cancer cell lines, TYMS copy number was, along with TYMS mRNA levels, negatively correlated with 5-FU sensitivity [27], suggesting that TYMS gene amplification may be associated with innate, in addition to acquired, 5-FU chemoresistance [27, 28]. All this, along with the relatively low frequency of allelic imbalance at the TYMS locus here observed in RCC tumors compared to other cancers (e.g. colon cancer [25]), raises the tempting speculation that genomic imbalances involving amplification, rather than deletion, of the TYMS locus may, at least in part, contribute to the relatively low efficacy of fluoropyrimidine-based chemotherapies in RCC malignancies. Although TYMS gene duplication has been found exclusively in colon cancer patients following 5-FU therapy [28], it cannot be excluded that highly proliferative tumors, such as RCC, are subjected to TYMS duplications prior to 5-FU treatment. TYMS duplication is directly correlated with high TYMS expression and 5-FU-resistance [26, 28], both features of which are typical of RCC malignancies. Nonetheless, given the low frequency of TYMS imbalance found in our patient population, additional factors are necessarily implicated in RCC chemoresistance.

Another possibility is that 18p deletions may include genes involved in the cell cycle regulation and control. Climent et al. [29] described a worse disease-free survival of breast cancer affected patients displaying a tumoral 18p chromosomal loss than those carrying the entire chromosome, suggesting the presence of one or more oncosuppressor genes mapping in 18p. More recently Rahman [30], reporting the transforming proprieties of the TYMS up-regulation in NIH/3T3 cells, proposed TYMS itself as a putative oncogene. This report, together with others showing high TYMS expression and/or activity in tumor samples [14], is compatible with the hypothesis that TYMS is involved in cancer proliferation and aggressiveness and its loss, rarely found in RCC masses, may be considered as a good prognosis factor.

In sum, by focusing on the TYMS polymorphic variants in renal cancer, we here provide evidence, to our knowledge, for the first time showing imbalance of 18p11.32 and 18p11.31 in renal cell carcinomas. As allelic imbalances involving TYMS locus may be an important variable affecting 5-FU responsiveness, further studies along this line may have the potential to provide for valuable surrogate markers of prognosis and 5-FU-based treatment outcomes in RCC malignancies.

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Conflict of interest statement None.

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