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Single nucleotide polymorphisms in the HIF-1α gene and chemoradiotherapy of locally advanced rectal cancer

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Abstract. The aim of this study was to investigate the predictive impact of polymorphisms in the HIF-1α gene on the response to chemoradiotherapy (CRT) in rectal cancer. This study included two cohorts of patients with locally advanced rectal cancer receiving long-course CRT. The HIF-1α C1772T (rs11549465), G1790A (rs11549467) and c*191T>C (rs2057482) polymorphisms were investigated in the test cohort (n=65), and HIF-1α c*191T>C was analysed in the validation cohort (n=198). No correlations were identified between the polymorphisms and clinicopathological factors. The HIF-1α C1772T and HIF-1α G1790A polymorphisms demonstrated no correlation with tumour response to CRT in the test cohort. The HIF-1α c*191T>C CC genotype was marginally associated with a higher rate of complete tumour response (P=0.05) in the test cohort, while the HIF-1α c*191T>C CC genotype was associated with a poor tumour response (P=0.03) in the validation cohort. In conclusion, these results suggest that HIF-1α polymorphisms have no value as predictors of response to neoadjuvant CRT in rectal cancer. The results of the HIF-1α c*191T>C in two cohorts differ and emphasise the importance of biomarker validation.

Introduction

Long-course chemoradiotherapy (CRT) followed by mesorectal excision is now considered as a standard treatment for locally advanced rectal cancer. Compared with radiation alone, CRT reduces the risk of local recurrence; however, the impact on overall survival is dubious (1). CRT is only beneficial to a subgroup of patients; thus, a more efficient patient selection for the combined treatment is required. A minor group of patients experiences complete tumour response, which may be verified by pathological examination of the surgical specimens. These patients may avoid surgery involving a permanent stoma (2,3). The current situation requires markers which are able to predict the complete tumour response with regards to patient selection.

Several markers of potentially predictive value have been studied, but to date, none have passed clinical validation, which is a hallmark for clinical routine application. Tumour hypoxia is a well-established phenomenon in the development of malignant tumours (4), and the importance of hypoxia to the effect of radiotherapy has been acknowledged for decades (5). Markers associated with hypoxia are evident candidates as predictors of response to CRT.

Hypoxia-inducible factor 1 (HIF-1) is a central protein involved in the cellular adaption to hypoxia. HIF-1 is a heterodimeric transcription factor consisting of HIF-1α and HIF-1β subunits (6), which induce the transcription of numerous genes that are necessary for the cellular response to hypoxia (7). Oxygen tension is essential for the activation of HIF-1. The presence of oxygen leads to the degradation of HIF-1α, while hypoxic conditions allow HIF-1α to bind to HIF-1β and become an active transcription-inducer (8). HIF-1α has been associated with aggressive tumour growth (4) and poor prognosis in several types of cancer, including colorectal cancer (9).

HIF-1 is an important mediator of hypoxia-induced radioresistance, and studies have demonstrated that cell lines with impaired HIF-1 activation ability are more sensitive to radiotherapy compared with cells with intact HIF-1 activation ability (10-12). Additionally, the *in vitro* response of tumour cells to 5-fluorouracil (5FU) appears to be dependent on HIF-1 (13).

Only a small number of studies have investigated the possible predictive value of HIF-1α in CRT of rectal cancer. In a previous study, we identified no correlation between immunohistochemical (IHC) expression of the HIF-1α protein and tumour response (14). Another study observed an association between the staining intensity of HIF-1α and poor response; however, this association was not significant when comparing HIF-1α positive tumours and HIF-1α negative tumours (15). The gene expression of HIF-1α was studied by Toiyama *et al* who identified an association between high HIF-1α gene expression and poor tumour response following short-course CRT (16), while Saigusa *et al* identified no correlation between

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HIF-1α gene expression and tumour response (17). To date, the results presented of HIF-1α protein and gene expression in relation to rectal cancer CRT have been inconclusive. The role of germline polymorphisms in the HIF-1α gene in relation to CRT remain to be explored.

The HIF-1α gene is located on chromosome 14 and a number of single nucleotide polymorphisms (SNPs) have been identified in the HIF-1α gene. The most frequently investigated polymorphisms are the C1772T (rs11549465), which comprises an amino acid change from proline to serine at position 582, and the G1790A (rs11549467), which results in the substitution of alanine with threonine at position 588. These polymorphic variants have demonstrated functional importance by increasing the transcriptional activity *in vitro* under normoxic and hypoxic conditions, and have been associated with increased microvessel density in patients with head and neck cancer (18).

Few studies have suggested a correlation between the above mentioned polymorphisms and the risk of colorectal cancer (19,20); while one study demonstrated an association between HIF-1α c*191T>C (rs2057482) and the risk of rectal cancer (21). No previous studies have investigated the possible importance of these polymorphisms for rectal cancer CRT.

The aim of this study was to investigate the predictive impact of germline C1772T, G1790A and c*191T>C polymorphisms in the HIF-1α gene on the response to CRT in locally advanced rectal cancer.

Patients and methods

Patients. The study included two cohorts treated with long-course CRT at the Department of Oncology (Vejle Hospital, Denmark). Inclusion criteria were patients with T3-T4 N0-N2 M0 histopathologically verified adenocarcinoma of the rectum, located <10 cm from the anal verge and with a distance of <5 mm to the mesorectal fascia. Pre-treatment staging was performed based on clinical examinations and magnetic resonance imaging (MRI) of the pelvis. Furthermore, computerised tomography (CT) of the chest and abdomen, or a chest X-ray and ultrasound of the liver were conducted to exclude distant metastases. Patients with available archival blood samples were included in this study.

The test cohort included 65 patients who were treated according to two previously published studies (22,23) with long-course CRT between 2002 and 2005, comprising conventional radiotherapy of 60 Gy/30 fractions and supplemented by a brachytherapy boost of 5 Gy. Concomitant chemotherapy consisting of 300 mg/m^2 UFT (uracil-tegafur; molar ratio 4:1) and 22.5 mg isovorin was administered to patients on treatment days.

The validation cohort included 203 patients who were treated according to a phase III randomised study between 2005 and 2009, comprising conventional CRT of 50.4 Gy/28 fractions with or without 10 Gy/2 fractions of brachytherapy. Concomitant chemotherapy was administered as previously described (24) Patient consent was obtained prior to inclusion in the studies (22-24) which were approved by the local ethics committee of The Regional Scientific Ethical Committee for Southern Denmark.

Table I. Clinicopathological parameters of rectal cancer patients.

Clinicopathological parameters	Test cohort, n (%)	Validation cohort, n (%)	P-value
Gender			
Male	45 (69)	126 (62)	NS
Female	20 (31)	77 (38)	
Age (years)			
Median	63.4	63.3	
Range	40.4-77.7	35.5-78.8	
cT classification			
3	62 (95)	168 (83)	0.01
4	3 (5)	35 (17)	
cN classification			
-	18 (28)	23 (11)	0.003
+	47 (72)	180 (89)	
TRG			
1	19 (29)	39 (19)	NS[a]
2	15 (23)	28 (14)	
3	29 (45)	120 (59)	
4	2 (3)	16 (8)	
ypN classification			
-	48 (74)	138 (68)	NS
+	17 (26)	65 (32)	
CPR (TRG 1 and ypN0)			
Yes	16 (25)	35 (17)	NS
No	49 (75)	168 (83)	
Total	65	203	

[a]TRG 1 vs. TRG 2-4. TRG, tumour regression grade; CPR, complete pathological response; NS, not significant (P>0.05).

Response evaluation. Total mesorectal excision surgery was scheduled 8 weeks following the end of CRT. Pathological response was evaluated in the surgical specimens according to Mandard's Tumour Regression Grade (TRG) (25). The endpoints of this study were complete tumour response, defined as the absence of viable residual tumour cells in the resected surgical specimens (TRG 1) and complete pathological response (CPR) defined as TRG 1 combined with no lymph node metastases (ypT0 and ypN0).

Analysis of SNPs. The investigated SNPs were selected as potentially clinically relevant polymorphisms based on previous studies (18-21,26-28). Genomic DNA was purified from whole blood using the NucloeSpin® Tissue method according to the manufacturer's instructions (NucleoSpin Tissue, Macherey-Nagel, Germany; Feb 2001/Rev. 02), or the Maxwell robot using 300 μl whole blood (elution was performed in 350 μl) and the Maxwell® 16 Blood DNA purification kit (AS1010; Promega, Madison, WI, USA), according to the manufacturer's instructions.

Table II. Distribution of the HIF-1α polymorphisms and the various genotypes.

	Test cohort		Validation cohort	
HIF-1α polymorphisms	n	% (95% CI)	n	% (95% CI)
HIF-1α c*191T>C				
CC	51	78 (67-87)	153	75 (69-81)
CT	13	20 (12-31)	41	20 (15-26)
TT	1	2 (0.3-8)	4	2 (1-5)
ND			5	2
HIF-1α C1772T				
CC	57	88 (78-94)		
CT	8	12 (6-22)		
TT	0			
HIF-1α G1790A				
GG	64	98 (92-100)		
GA	1	2 (0.3-8)		
AA	0			
Total	65		203	

Sum of the percentages does not always equal 100% due to the rounding of data. HIF-1α, hypoxia-inducible factor-1α; CI, confidence interval; ND, not determined.

In the test cohort, the SNP analyses were conducted in-house. The HIF-1α c*191T>C was analysed using the Taqman assay (c_8549084_20) with 2 μl DNA and genotyping master mix in a total volume of 20 μl, using the 7900 HT Real-Time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) on a standard programme of genotyping.

The HIF-1α C1772T and G1790A were analysed by sequencing. Initially, PCR was performed with 2 μl DNA, 0.5 μM primer, 2.0 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphate (dNTP) and 0.04 units Taq Gold with buffer (Applied Biosystems), in a total volume of 25 μl with cycling conditions as follows: 95˚C for 10 min, and 40 cycles at 95˚C for 40 sec, 54˚C for 40 sec and 72˚C for 40 sec, followed by 72˚C for 10 min. The PCR products were treated with ExoSAP-IT (USB Corp., Cleveland, OH, USA), to remove unused primers and nucleotides. PCR sequencing was then conducted with 1 μl PCR product, 1.5 μl Big Dye sequencing buffer, 1 μl terminator RR mix, and 1 μl 1.6 μM forward or reverse primer, in a total volume of 10 μl with cycling conditions as follows: 96˚C for 1 min, and 25 cycles at 96˚C for 10 sec, 50˚C for 5 sec and 60˚C for 4 min.

The primers (forward, 5'-GAC TTG GAG ATG TTA GCT CCC TA-3'; reverse, 5'-TTC TTG TAT TTG AGT CTG CTG GA-3') were designed in-house. After 45 μl SAM solution and 10 μl Big Dye Terminator were added to the 10 μl sequencing PCR product, the sequencing product was analysed using a 3130 Genetic Analyzer (Applied Biosystems). Sequences were analysed using Sequencing Analysis Software version 5.2 (Applied Biosystems). In each method, controls were analysed alongside the samples.

In the validation cohort, purified DNA was sent to KBioscience (Hoddesdon, Hertfordshire, UK; http://www.kbioscience.co.uk) who conducted the SNP genotyping using a KASPar genotyping system, designed and validated by KBioscience. Data were inspected using their SNPviewer PC tool.

Statistical analysis. Fisher's exact test was used to analyse the association between clinicopathological parameters, TRG and the polymorphisms, as well as the differences in these parameters between the two cohorts. Confidence intervals (CIs) of the proportions of polymorphisms were calculated using the Wilson score method. All tests were two-sided and $P<0.05$ was considered to indicate a statistically significant difference. NCSS 2007 statistical software (NCSS, Kaysville, UT, USA) was used for all analyses.

Results

Patient characteristics. Clinicopathological characteristics of the two cohorts are presented in Table I. The majority of patients in both cohorts had a T3 tumour, while there was a higher frequency of T4 tumours in the validation cohort ($P=0.01$). The rate of patients without clinical lymph node metastases also varied ($P=0.003$), but there was no difference with regard to complete tumour response (TRG 1) or frequency of CPR between the two cohorts.

Polymorphisms. HIF-1α c*191T>C, HIF-1α C1772T and HIF-1α G1790A polymorphisms were analysed in 65 patients in the test cohort, and the HIF-1α c*191T>C polymorphism was successfully analysed in 198 of the 203 patients in the validation cohort.

The distribution of the three polymorphisms in the HIF1α gene is shown in Table II. All polymorphisms were in agreement

Table III. TRG 1 and CPR according to HIF-1α c*191T>C.

Polymorphism and genotype	TRG 1		TRG 2-4			CPR+		CPR-		
	n	%	n	%	P-value	n	%	n	%	P-value
Test cohort										
HIF-1α c*191T>C CC	18	35	33	65	0.05	15	29	36	71	0.16
HIF-1α c*191T>C CT/TT	1	7	13	93		1	7	13	93	
Total	19		46			16		49		
Validation cohort										
HIF-1α c*191T>C CC	25	16	128	84	0.03	24	16	129	84	0.19
HIF-1α c*191T>C CT/TT	14	31	31	69		11	24	34	76	
Total	39		159			35		163		

TRG, tumour regression grade; CPR, complete pathological response; HIF-1α, hypoxia-inducible factor-1α.

with the Hardy Weinberg equilibrium. The homozygous TT genotype was rare in the HIF-1α c*191T>C and HIF-1α C1772T polymorphisms. The same applied to the GA and AA genotypes of the HIF-1α G1790A polymorphism. No association was identified between the three polymorphisms and clinicopathological factors. Additionally, there was no correlation between the HIF-1α C1772T or HIF-1α G1790A polymorphism and response to CRT as measured by TRG and CPR in the test cohort, and consequently they were not analysed in the validation cohort.

Table III shows the association of the HIF-1α c*191T>C polymorphism with TRG and CPR. In the test cohort, a high rate of complete tumour regression was observed in patients with the CC homozygous genotype (35%) compared with the CT/TT genotype (7%) (P=0.05). Similarly, CPR was observed in 29 and 7% of patients with CC and CT/TT genotype, respectively; however, this was not significant (P=0.16). In the validation cohort there appeared to be a statistically significantly lower rate of TRG 1 in patients with the CC genotype (16%) compared with the CT/TT genotype (31%; P=0.03). Similarly, CPR was observed in 16 and 24% of patients with the CC and CT/TT genotype, respectively; however, this was not significant (P=0.19).

Discussion

Numerous biomarkers have been studied in the search for a prediction of CRT response in rectal cancer. To date, none have proven to be valid for clinical application. SNPs are attractive due to their stable nature and inexpensive analysis costs. Additionally, blood cells are easily accessible material.

To the best of our knowledge, the polymorphisms of the HIF-1α gene have not been studied with regard to CRT; however, HIF-1α protein expression has been subject to investigation in a number of malignant tumour types. In patients with head and neck cancer, previous studies have indicated a predictive importance of IHC HIF-1α protein expression in relation to radiotherapy (29-31), which translated into poor survival. Similarly, IHC HIF-1α expression has been associated with poor outcome following radical radiotherapy in patients with cancer of the cervix uteri (32,33).

Based on previous studies, it is justified to investigate the possible predictive value of HIF-1α in CRT of rectal cancer. However, the few studies on IHC expression of HIF-1α and tumour response, including recent data from our own group, have revealed conflicting results, and the same applies to studies investigating HIF-1α gene expression levels of the marker (14-17). Therefore, in the present study, we investigated polymorphisms of the HIF-α gene in patients with rectal cancer.

In contrast to previous data suggesting a correlation between HIF-1α polymorphisms and clinicopathological parameters in colorectal cancer, we did not identify a correlation between the HIF-1α polymorphisms and any of the pretreatment parameters (26,28). Knechtel *et al* reported that the HIF-1α G1790A GA/AA genotype was associated with localisation in the rectum and a higher T category (28); however, similar to Kang *et al*, the authors identified no correlation between HIF-1α C1772T and clinicopathological parameters (19). With a small HIF-1α G1790A GA/AA frequency of 11/341 in their population and 1/65 in our cohort, it is difficult to make a qualified comparison. Fransen *et al* identified an association between ulcerative tumours and the variant allele of the HIF-1α C1772T or HIF-1α G1790A in 198 CRC patients; however, no correlation between the other clinicopathological parameters was observed (26).

In the present study, we identified a marginal association between T-containing genotypes of HIF-1α c*191T>C and poor response to CRT in the test cohort. However, this could not be confirmed in the validation cohort, which demonstrated a significantly opposing effect. The results presented in this study underline the importance of validation studies. The literature on biomarkers is characterised by a high number of small 'positive' studies, which are never validated in new prospective patient materials, and the risk of small 'positive' studies demonstrating a false positive is extremely high (34).

The two cohorts in this study varied with regards to the pre-treatment cT/cN category and the type of radiotherapy administered. The test cohort included patients from two previously published studies of which one solely included T3 tumours. This explains the differences in the cT category. Additionally, a difference in lymph node metastasis pre-treatment evaluation was observed between the two cohorts, but

no difference was observed in the lymph node metastasis post-treatment pathological evaluation. All patients of the test cohort received brachytherapy, while only half of the patients in the validation cohort received brachytherapy. However, in the validation cohort there was no difference in the association of the polymorphism and the response to CRT between patients receiving brachytherapy and those who did not. These minor differences between the cohorts are unlikely to explain the various results on tumour response in the two cohorts. Therefore, a clinically relevant predictive role of the polymorphisms with regards to neoadjuvant CRT of rectal cancer is dubious.

In conclusion, we were unable to find any predictive value of the HIF-1α C1772T, HIF-1α G1790A and HIF-1α c*191T>C polymorphisms with regards to tumour response following neoadjuvant CRT of locally advanced rectal cancer.

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