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ORIGINAL ARTICLE

The Expression of Checkpoint and DNA Repair Genes in Head and Neck Cancer as Possible Predictive Factors

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Abstract DNA damage response failure may influence the efficacy of DNA-damaging treatments. We determined the expression of 16 genes involved in distinct DNA damage response pathways, in association with the response to standard therapy. Twenty patients with locoregionally advanced, squamous cell head and neck carcinoma were enrolled. The treatment included induction chemotherapy (iChT) with docetaxel, cisplatin and 5-fluorouracil followed by concomitant chemoradiotherapy (ChRT) or radiotherapy (RT) alone. The volumetric metabolic therapeutic response was determined by [18F]FDG-PET/CT. In the tumor and matched normal tissues collected before treatment, the gene expressions were examined via the quantitative real-time polymerase chain reaction (qRT-PCR). The down-regulation of *TP53* was apparently associated with a poor response to iChT, its up-regulation with complete regression in 2 cases. 7 cases with down-regulated *REV1* expression showed complete regression after ChRT/RT, while 1 case with *REV1* overexpression was resistant to RT. The overexpression of *WRN* was an independent predictor of tumor relapse. Our results suggest that an altered expression of *REV1* predicts sensitivity to RT, while *WRN* overexpression is an unfavorable prognostic factor.

Keywords Head and neck cancer · Chemosensitivity · Radiosensitivity · DNA damage response · Gene expression

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Introduction

Head and neck cancer (HNC) is significant in Europe as regards both incidence and mortality [1]. In about 60 % of all cases presenting with advanced disease, induction chemotherapy (iChT), typically with the TPF regimen (docetaxel, cisplatin and 5-fluorouracil [5-FU]), followed by radiotherapy (RT) alone or with concurrent cisplatin is used with the aim of organ preservation [2–4]. In the development of HNC, environmental factors such as alcohol and tobacco abuse and HPV infection are implicated. Despite advances in our knowledge of its epidemiology and pathogenesis, the survival rate of HNC is very poor mostly in the locally advanced cases. However, the improving understanding of DNA damage response is providing new possibility to predict therapy response and new targets for cancer management [5, 6].

Table 1 Roles of investigated genes in DNA damage response

Genes	Function of protein encoded	DDR	Damage
RAD54L RAD51	Form and stabilize nucleoprotein filament	HR	- interstrand crosslinks
			- stalled replication fork
			- replication-associated DSBs
ERCC4	Endonuclease, cleaves injury-containing DNA fragment at positions 3′ and 5’	NER	- helix-distorting adducts or base modifications
			- crosslinks
PMS2	Forms heterodimer, MutLα-complex and triggering checkpoint signals and apoptosis	MMR	- base-pairing errors
			- small single-stranded
			- DNA loop
WRN BLM	RecQ helicases, DNA-unwinding enzymes	NER, MMR, BER, HR, NHEJ	- base mismatch
			- DSBs
			- replication errors caused by stalled replication forks
			- other anomalous DNA structures
REV1	Coordinates and facilitates replacement of replicative polymerase with TLS polymerase	TLS (can substitute HR)	- base damage
			- DNA adducts
			- crosslinks
SPRTN	Prevents inactivation by deubiquitination of PCNA		- blocked replication fork
RAD17	With RFC forms a clamp loader complex	G1/S and G2/M checkpoints	- ssDNA (RPA-coated)
RAD1	Forms heterotrimeric complex Rad9-Hus1-Rad1		- resected DSBs
ATRIP ATR CHEK1 CHEK2	Kinase-dependent pathways responsible for phosphorylation, activation and accumulation of p53		- DSBs
TP53	Depending on cell type and severity of damage, promotes cell cycle arrest, apoptosis or DNA repair		- various damage in the DNA
MAD2L1	Forms the mitotic checkpoint complex	M-phase checkpoint	- inadequate attachment of spindle microtubules to the kinetochore

BER base excision repair, *DDR* DNA damage response, *DSBs* double-strand breaks, *HR* homologous recombination, *MMR* mismatch repair, *NHEJ* non-homologous end-joining, *NER* nucleotide excision repair, *PCNA* proliferating cell nuclear antigen, *RPA* replication protein A, *RFC* replication factor C, *ssDNA* single-strand DNA, *TLS* translesion synthesis

We designed a pilot prospective study to ascertain the expression of 16 DNA damage response genes in individual HNC samples, together with the efficacy of therapy and the outcome. The selected genes [5–12] (Table 1) are involved in the detection or repair of DNA damage caused by conventional treatment (docetaxel, cisplatin, 5-FU and RT) of HNC. We aimed at the identification of certain gene abnormalities as possible predictive factors of therapeutic sensitivity.

Material and Methods

The study was approved by the Institutional Review Board of the University of Szeged, and all enrolled patients gave their written informed consent to participation in the study.

Patient Characteristics and Management; Sample Collection

Eligible patients had histologically confirmed, non-metastatic, technically/oncologically inoperable (or unresectable without loss of function), locoregionally advanced squamous cell carcinoma (TNM stage III, IV) of the head and neck. Exclusion criteria were previous ChT, RT, surgery for the head and neck cancer, any contraindication of the planned therapy or an ECOG status >1. The patient management is outlined in Fig. 1.

Tumorous and normal mucosal samples (from the contralateral healthy anatomical region) collected during panendoscopy were cut into two: one half for routine verification, and one for gene expression studies. They were stored in RNAlater® solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA) overnight at

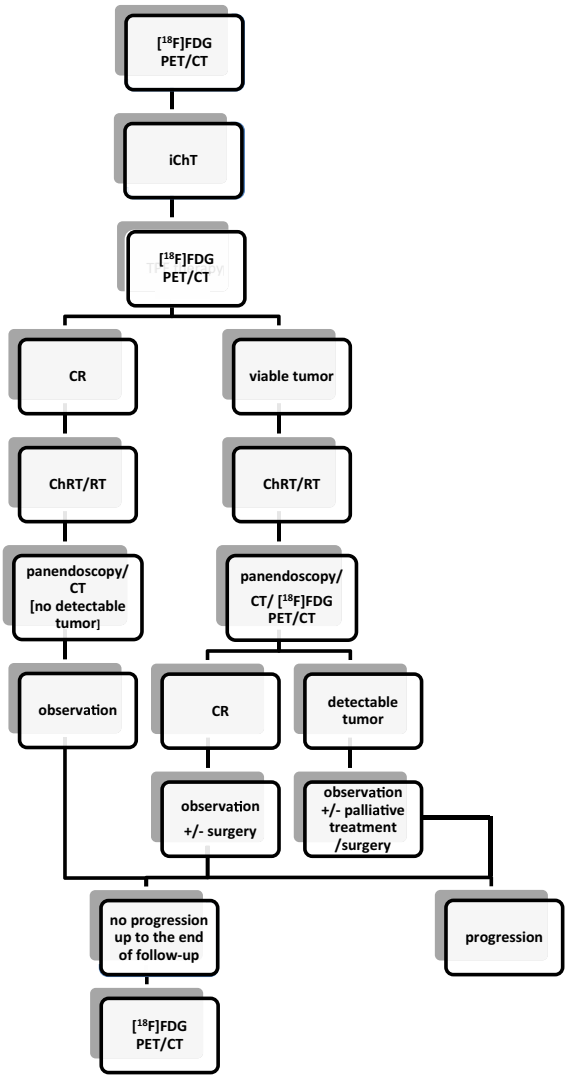

Fig. 1 Consort diagram of patient management

CR: complete regression, CT: computed tomography; ChRT: chemoradiotherapy; iChT: induction chemotherapy; RT: radiotherapy

4 °C and then solution-free at −80 °C until RNA isolation.

PET/CT with [^{18}F]FDG was performed within 2 weeks before the start and 3–6 weeks after the completion of the iChT. Tumor volumes after 3D reconstruction were extracted from the Oncentra Masterplan® RT planning system (Elekta, Stockholm, Sweden).

The tumor response was evaluated via the WHO classification; cases without viable or detectable tumor were classified as complete regression (CR) [13].

Treatments

The patients received 3 cycles of iChT: docetaxel (75 mg/m^2) day 1, cisplatin (50 mg/m^2) days 1–2, 5-FU (1000 mg/m^2) days 1–5, q3 weeks (TPF regimen).

Subsequently, 5–8 weeks later, concurrent ChRT with weekly cisplatin (30 mg/m^2) was scheduled. 3D conformal RT planning was based on PET/CT images, applying the ConPas irradiation technique [14] with individually shaped 6-MV and 15-MV photon fields. The aim was a mean dose of 28 × 1.8-Gy plus 12 × 1.8-Gy boost.

End-Points

The primary end-point of the study was the evaluation of the response to 3 cycles of iChT, and the association between the therapy response and the examined gene status or initial tumor volume.

The secondary end-point was the response to ChRT or RT following iChT and survival, in association with the gene

Table 2 Specification of primer pairs for selected and reference genes

Gene	Forward	Reverse	Reference sequence	Forward primer location	Reverse primer location	Amplicon length
RAD54L	5′-AGAAAGGAAGTGTTGGTCTGG-3’	5′-AGGGCTTGGTAAGTCTGATTC-3’	NM_001142548.1	971–991	1029–1049	78 bp
RAD51	5′-GATCTGTCATACGCTAGCTGTC-3’	5′-ACCATACCTCTCAGCCACT-3’	NM_001164269.1	552–573	666–684	132 bp
ERCC4	5′-GCTATACAGACTGCTATACTGGAC-3’	5′-AAAGGATCCAGATAATGGCGG-3’	NM_005236.2	679–702	807–827	148 bp
PMS2	5′-GCCTCATTCCTTTTGTTCAGC-3’	5′-AACTCCTTCCAACTCCATGC-3’	NM_000535.5	800–820	918–937	137 bp
WRN	5′-TGCTAGTGATTGCTCTTTCCTG-3’	5′-CTTTGCCAAGTTTCCCTCTATTG-3’	NM_000553.4	962–983	1055–1077	115 bp
BLM	5′-TGCTCTTGCTTACCATGCTG-3’	5′-GAATCACAAATCGCACGTCC-3’	NM_000057.2	2839–2858	2965–2984	145 bp
REV1	5′-ATCATGGTACGAAAGCCTGG-3’	5′-CATGTTTAGCATCGCCTTTCC-3’	NM_001037872.1	2478–2492	2598–2618	140 bp
SPRTN	5′-CCTTTTGAAGTTGAGGCCAAG-3’	5′-TCAGGCTGTTGATGCGATG-3’	NM_001010984.2	733–753	863–881	148 bp
RAD17	5′-CCAGAAACTCAGCATGAACTTG-3’	5′-TCGTTGTCTTTCCACATCCAG-3’	NM_001278622.1	793–814	920–940	147 bp
RAD1	5′-TCTCCTGACAAGCCTTATTTCAG-3’	5′-TGTATCTGTTGACTTGGGTCTG-3’	NM_002853.3	873–895	978–999	126 bp
ATRIP	5′-CAGTGACAAGGAAAAGGAATTCTC-3’	5′-GACATGGGAAACAGAGGGAG-3’	NM_001271022.1	448–471	576–595	147 bp
ATR	5′-GCCAAACTCAACAGGAAAACC-3’	5′-GCTCTTTTGGTTCATGTCCAC-3’	NM_001184.3	1360–1380	1482–1502	142 bp
CHEK1	5′-AGTTGATGTTTGGTCCTGTGG-3’	5′-CTGTCACTGGGTTGGTCC-3’	NM_001114121.2	1461–1481	1521–1538	77 bp
CHEK2	5′-GCGCCTGAAGTTCTTGTTTC-3’	5′-GTCCTATGCTCAGAGAAAGGTG-3’	NM_001005735.1	1375–1394	1475–1496	121 bp
MAD2L1	5′-GACAGATCACAGCTACGGTG-3’	5′-GGCGGACTTCCTCAGAATTG-3’	NM_002358.3	522–541	652–671	149 bp
TP53	5′-GCCATCTACAAGCAGTCACA-3’	5′-TCATCCAAATACTCCACACGC-3’	NM_000546.5	683–702	805–825	142 bp
GAPDH	5′-AATCCCATCACCATCTTCCAG-3’	5′-AAATGAGCCCCAGCCTTC-3’	NM_001256799.1	412–432	516–533	121 bp
SDHA	5′-TGGTTGTCTTTGGTCGGG-3’	5′-GCGTTTGGTTTAATTGGAGGG-3’	NM_004168.2	1494–1511	1558–1578	84 bp

status or initial tumor volume. Survival was also analyzed as concerns the response to therapy.

HPV Immunohistochemistry

Tissue microarrays were constructed from formalin-fixed and paraffin-embedded tissue blocks, and slides were made as described [15]. For immunostaining the EnVision® FLEX kit (Dako, Glostrup, Denmark) was used with an automatic staining machine (Dako Autostainer Plus, Dako, Glostrup, Denmark). The sections were incubated with HPV16 L1 antibody (clone CAMVIR-1) (Santa Cruz Biotech, Dallas, Texas, USA) 1:800. The density of immunostaining was scored (0–3) by two independent examiners.

RNA Extraction and cDNA Synthesis

Tissue samples were homogenized in Tri Reagent® (Sigma-Aldrich, St. Louis, Missouri, USA) solution with an IKA Ultra-Turrax T8® (IKA, Staufen, Germany) homogenizer. Total RNA was isolated with the Tri Reagent® extraction kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions. The RNA degradation level was checked on 0.8 % agarose gel and the quantity was determined with a Qubit® 2.0 fluorimeter (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA was synthesized from 1 μg DNase-treated RNA as template with 200 U/μl M-MuLV reverse transcriptase (Fermentas, Waltham, Massachusetts, USA) and random hexanucleotide primers in a 20-μl reaction volume.

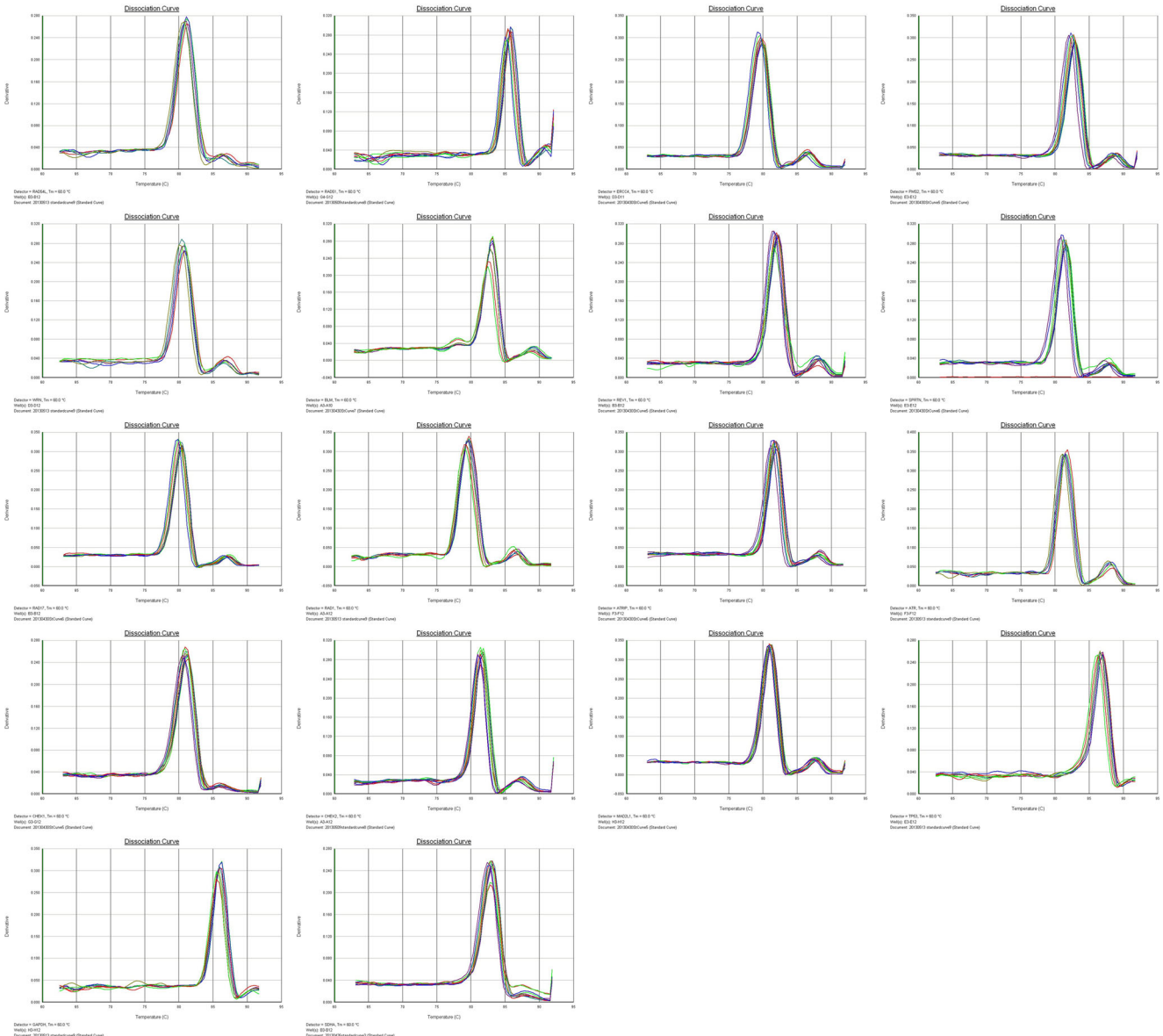

Fig. 2 Dissociation curves

Table 3 Tumor characteristics [$n = 20$]

Initial tumor volume (mean ± SD, cm^3)	23.9 ± 19.0
TNM Stage	
III (n [%])	6 [30.0]
IV (n [%])	14 [70.0]
Localization	
Oropharynx (n [%])	11 [55.0]
Hypopharynx (n [%])	7 [35.0]
Larynx (n [%])	2 [10.0]
Histology	
Well-differentiated (n [%])	1 [5.0]
Moderately-differentiated (n [%])	12 [60.0]
Poorly-differentiated (n [%])	7 [35.0]
HPV16 infection	
Positive (n [%])	2 [10.0]
Negative (n [%])	18 [90.0]

HPV Human papilloma virus, *n* No. of patients, *SD* standard deviation

Transcription was performed in duplicate from each RNA template.

Quantitative Real-Time PCR

qRT-PCR was performed via the SYBR Green approach on ABI PRISM 7500 Real-time PCR® (Applied Biosystems, Waltham, Massachusetts, USA). Primer pairs are listed in Table 2. Primer pairs for selected genes were designed using the Primer3® software [16] implanted in Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were designed to amplify all the known isoforms of the respective genes. The specificity of the primers was checked by performing BLAST search. To avoid product amplification from genomic DNA, one primer from each pair was selected to span an exon junction. Primers were tested at 4 different concentrations (200, 300, 400 and 500 nM) to ensure optimal assay performance. The 300 nM primer concentration was chosen for further reactions.

Reaction efficiencies were calculated with the standard curve method. Specificity was confirmed by the presence of a single peak at the expected temperature on melting curve analysis (Fig. 2).

The PCR reaction was carried out in a 10-µl reaction mixture containing 0.4 µl template cDNA, 2 µl mixture of forward and reverse primers (3 µM each), 5 µl PCRBiO qPCR Master Mix (2X)® (Bio-Rad, Hercules, California, USA) and nuclease-free water. The cycling conditions: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 62 °C for 33 s. The negative control for this experiment was the combination of all reagents without template.

Two parallel PCR reactions were performed on each cDNA template. For normalization, the *GAPDH* and *SDHA* genes were chosen [17] and the comparative C_T (Threshold cycle)

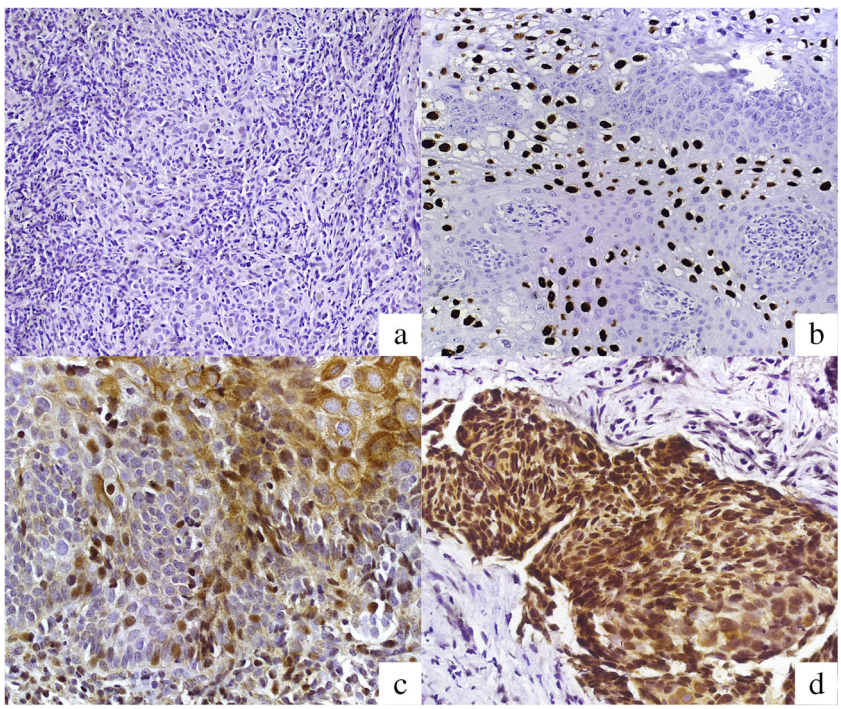

Fig. 3 HPV16 immunohistochemistry examination **a** negative staining (20× magnification); **b** positive control (20× magnification), verruca vulgaris; (**c**, **d**) positive staining (40× magnification) in 2 samples

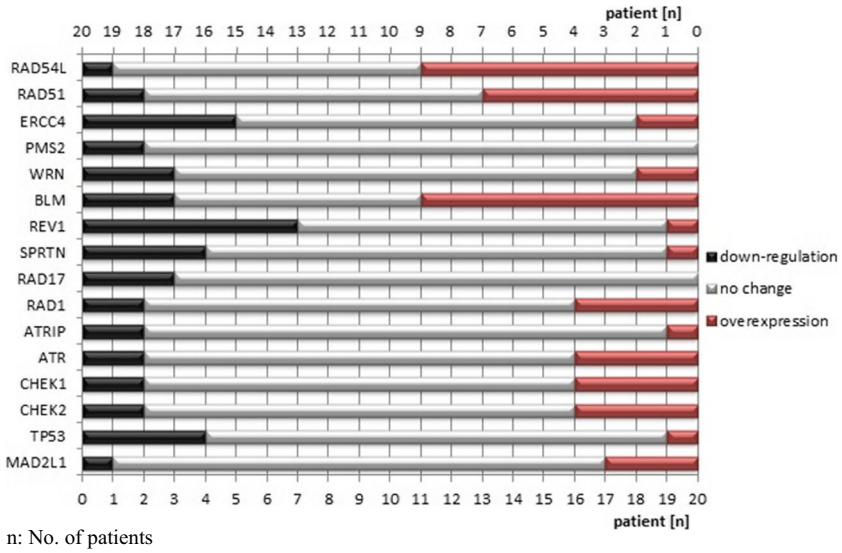

Fig. 4 Distribution of gene expression levels among cancer samples

n: No. of patients

method was used to calculate the relative changes in gene expression. The fold change in expression was ascertained with the formula $2^{-\Delta\Delta C}{}_T$; a > 2.0 or <0.5-fold change was accepted as relevant.

Statistical Analysis

The independent samples t-test was used to compare initial tumor volumes between groups. The influence of gene expression or therapy response on RFS and PFS and the effects of initial tumor volume (the cutoff value was the median of the initial tumor volume; >18.8 cm^3 vs. ≤18.8 cm^3) and *WRN* gene expression on PFS were estimated with the Kaplan-Meier method. IBM SPSS version 20.0 for Windows (IBM Corp., Armonk, New York, USA) was utilized for statistical analysis.

Results

Between March 2009 and April 2011, 20 tumors and matched normal tissues were collected from 20 (15 male and 5 female) patients. Tumor characteristics are presented in Table 3.

All the patients underwent the planned iChT. Two patients (with CR) refused any further treatment, 13 completed ChRT and 5 received RT only, due to the decline in their performance status. The median number of cisplatin cycles during ChRT was 5 (3–7). 13 patients completed the RT protocol as planned, while 5 patients had a dose reduction to a median dose of 67.5 (65.2–72.0) Gy. No tumor progression was detected in any of the patients during the therapy.

HPV16 immunohistochemistry test was positive in 2/20 (10 %) cases (Table 3, Fig. 3).

As regards the qRT-PCR results, gene overexpression was more frequent than down-regulation (Fig. 4). Decreased *REV1* ($n = 7$) and *ERCC4* ($n = 5$) transcription activity, and *BLM* and *RAD54L* overexpression (9 cases each) were the most frequent anomalies. Although each examined gene was down-regulated in at least one patient, *PMS2* and *RAD17* overexpression did not occur in any case. In most cases there was no difference in mRNA level between normal and tumor tissues.

Primary End-Point: Gene Expression Profile and Response to iChT

After the iChT, CR was detected in 6 cases (30.0 %), while [^{18}F]FDG-PET/CT indicated viable tumor remaining in 14 patients (70.0 %). The only difference in gene expression profile between the two groups was the overexpression of *TP53* in 2 cases in the CR group, and the down-regulation of *TP53* in 4 cases in the poor therapy response group (Table 4).

When the initial tumor volume was smaller, the response to therapy was better ($p = 0.041$) (Table 5).

Secondary End-Points: Gene Expression Profile and Response to CRT/RT; Survival

In the 14 patients with residual tumor after iChT, the response to second-line therapy was assessed. 4 still had a tumor residue despite ChRT/RT treatment. All the cases with down-regulated *REV1* ($n = 7$) responded with complete tumor regression to ChRT/RT, while the patient with a high *REV1* level

was resistant to the therapy. A larger post-iChT tumor volume was associated with a poorer response to ChRT/RT ($p = 0.035$) (Table 5).

Kaplan-Meier analysis revealed that the larger the initial tumor volume before iChT, the greater the risk of progression ($p = 0.009$) (Fig. 5a).

Relapse Status and Survival

The median follow-up time was 34.9 (range: 7.9–57.5) months. The RFS in the patients who achieved CR ($n = 16$), was 26.4 (range: 0.9–50.1) months. The median PFS for the overall patient population was 29.6 (range: 7.9–57.5) months, while in those who did or did not achieve CR it was 31.9 (95 % CI: 14.4–48.8) and 11.4 (95 % CI: 5.5–16.9) months, respectively. There was no significant difference in RFS or PFS between those who became tumor-free as a result of iChT or ChRT/RT (Fig. 5b and c). Patients with residual tumor after treatments had the worst PFS ($p < 0.001$; Fig. 5d).

Kaplan-Meier analysis (Fig. 5e) demonstrated that the median PFS was longer in the cases where the *WRN* gene expression was down-regulated (46.1, 95 % CI: 33.2–59.0) or unchanged (28.8, 95 % CI: 19.7–37.9) as compared with an overexpressed (7.92, 95 % CI: 5.3–10.5) *WRN* status ($p = 0.001$). Changes in the expression of other genes were not related to survival.

Discussion

The failure to respond to DNA damage that is prevalent in most cancers influences the sensitivity to therapy. We set out to identify new biomarkers that could predict the therapeutic response in conventionally treated HNC. For this we analyzed the differences in transcriptional activity of various DNA repair and cell cycle regulator genes between normal and HNC tissues.

Our study furnished an unexpected and intriguing finding: the increased radiosensitivity of tumors with low *REV1* gene expression and the radioresistance of a tumor with upregulated *REV1*. *REV1* promotes the tolerance and repair of DNA damage (e.g. interstrand crosslinks or base damage), and facilitates acquired therapy resistance [18, 19], while *REV1* loss increases sensitivity to cytostatic agents [20]. In line with our findings, disruption of *REV1* caused hypersensitivity to various genotoxic treatments, including ionizing radiation, in chicken B-lymphocytes [21]. These results suggest that *REV1* predicts and could be a biomarker of radiosensitivity. Other biomarkers of radiosensitivity have been described. *KU80* has been validated as an independent predictive factor of radioresistance in HNC [22]. Among >500 genes, the expression of *NM-23* (also involved in DNA repair) was related

Table 4 Association between response to iChT and mRNA expression of studied genes

		CR n [%]	Residual tumor n [%]
RAD54L	Down-regulation	0 [0.0]	1 [7.1]
	Overexpression	3 [50.0]	6 [42.9]
	No change	3 [50.0]	7 [50.0]
RAD51	Down-regulation	0 [0.0]	2 [14.3]
	Overexpression	2 [33.3]	5 [35.7]
	No change	4 [66.7]	7 [50.0]
ERCC4	Down-regulation	1 [16.7]	4 [28.6]
	Overexpression	0 [0.0]	2 [14.3]
	No change	5 [83.3]	8 [57.1]
PMS2	Down-regulation	0 [0.0]	2 [14.3]
	Overexpression	0 [0.0]	0 [0.0]
	No change	6 [100.0]	12 [85.7]
WRN	Down-regulation	0 [0.0]	3 [21.5]
	Overexpression	1 [16.7]	1 [7.1]
	No change	5 [83.3]	10 [71.4]
BLM	Down-regulation	0 [0.0]	3 [21.4]
	Overexpression	3 [50.0]	6 [42.9]
	No change	3 [50.0]	5 [35.7]
REV1	Down-regulation	0 [0.0]	7 [50.0]
	Overexpression	0 [0.0]	1 [7.1]
	No change	6 [100.0]	6 [42.9]
SPRTN	Down-regulation	2 [33.3]	2 [14.3]
	Overexpression	0 [0.0]	1 [7.1]
	No change	4 [66.7]	11 [78.6]
RAD17	Down-regulation	0 [0.0]	3 [21.4]
	Overexpression	0 [0.0]	0 [0.0]
	No change	6 [100.0]	11 [78.6]
RAD1	Down-regulation	0 [0.0]	2 [14.3]
	Overexpression	0 [0.0]	4 [28.6]
	No change	6 [100.0]	8 [57.1]
ATRIP	Down-regulation	0 [0.0]	2 [14.3]
	Overexpression	0 [0.0]	1 [7.1]
	No change	6 [100.0]	11 [78.6]
ATR	Down-regulation	0 [0.0]	2 [14.3]
	Overexpression	1 [16.7]	3 [21.4]
	No change	5 [83.3]	9 [64.3]
CHEK1	Down-regulation	0 [0.0]	2 [14.3]
	Overexpression	1 [16.7]	3 [21.4]
	No change	5 [83.3]	9 [64.3]
CHEK2	Down-regulation	0 [0.0]	2 [14.3]
	Overexpression	0 [0.0]	4 [28.6]
	No change	6 [100.0]	8 [57.1]
MAD2L1	Down-regulation	0 [0.0]	1 [7.1]
	Overexpression	0 [0.0]	3 [21.5]
	No change	6 [100.0]	10 [71.4]
TP53	Down-regulation	0 [0.0]	4 [28.6]
	Overexpression	2 [33.3]	0 [0.0]
	No change	4 [66.7]	10 [71.4]

CR complete regression, *n* No. of patients

Table 5 Tumor volume changes after iChT and ChRT/RT in cases showing CR or PR/SD

Intervention		Tumor volume* (cm^3) (mean ± SD, range)			*p* value
iChT		CR (*n* = 6)	Residual tumor (*n* = 14)		
	Before	14.36 ± 5.70 [3.67–18.74]	27.97 ± 11.30 [5.61–93.64]		0.041
	After	0	13.38 ± 6.09 [0.96–58.65]		-
ChRT/RT		0	CR (*n* = 10)	Residual tumor (*n* = 4)	p
	Before		6.47 ± 4.72 [0.98–16.39]	30.67 ± 15.54 [0.96–58.65]	0.035
	After		0	20.62 ± 13.52 [8.50–35.20]	-

ChRT chemoradiotherapy, *CR* complete regression, *iChT* induction chemotherapy, *n* No. of patients, *RT* radiotherapy, *SD* standard deviation

*calculated from PET/CT scans

The *p*-values were calculated with independent samples t-test

to the acquisition of radioresistance [23]. Both *KU80* and *NM-23* were related to poor prognosis. We do not think that the expression of the *REV1* gene is the only determinant of ChRT sensitivity, but our findings raise the question of whether ChRT/RT could be the first line of treatment in cases with low *REV1* expression. Obviously, more investigations are needed to confirm this possibility.

Survival analyses indicated that complete disappearance of the tumor is the most important predictor of a good outcome. In our patient cohort, the CR group had a significantly longer PFS relative to the non-CR group. The efficacy of therapy was followed by [^{18}F]FDG-PET/CT, which visualizes the metabolically active tumor tissue before and after therapy, and quantitates tumor volume changes data sensitively and accurately [24, 25]. The CR rates after iChT and ChRT/RT were similar to those reported in previous studies [3, 26, 27]. Our findings are in accordance with literature data in that smaller tumors were more likely to respond with CR or a better outcome [27, 28].

p53 is the guardian of the genome [29]. Numerous clinical data demonstrate that the presence of wild-type p53 in the tumor is a favorable prognostic marker [30, 31]: through apoptosis induction, it is a significant factor in tumor susceptibility to treatment [32]. In our study, a low *TP53* expression favored chemoresistance, but did not cause resistance to ChRT/RT applied as second-line treatment. These findings could be explained by the activation of p53-independent apoptotic pathways [33–35] and are consistent with laboratory data which suggest that the presence of the *TP53* mutation does not exclude the effectiveness of radiotherapy [36, 37]. Furthermore, the loss of p53 was related to increased radiosensitivity in a report where p53 disruption sensitized human colorectal cancer cells to doxorubicin and radiation, but not to 5-FU [38]. Our findings support the earlier conclusion that p53-dependent apoptosis is significant in the therapy response in only a small fraction of malignancies, and highlight the need for parallel investigations of other apoptotic mediators [29]. The consequences of the impaired function of mutated p53 depend on the biological and biochemical properties of the gene product [39]. It is important that HPV infection (commonly detected in HNC) could promote the proteolytic breakdown and impaired function of p53 [25, 39, 40]. In our study, among 20 samples only 2 were positive for HPV16 infection. In contrast, data extracted from patient files indicated that alcohol and/or tobacco abuse were present as etiological factors in the majority of the cases (including the 2 with HPV16 positivity). The small number of samples limits the analysis of these factors in relation with gene expression status or therapy response.

WRN belongs in the SF2 superfamily of helicases, and plays a crucial role in DNA recombination, replication, repair and transcription. Like other RecQ helicases, *WRN* can salvage cancer cells from DNA damage-induced cell death and its expression is upregulated in highly proliferating tumors [9, 41]. Among the 16 genes we investigated, only *WRN* displayed an independent association with survival. We believe that the high mRNA expression of *WRN* is simply a reflection of the aggressive nature of the cancer. Although we failed to demonstrate the role of *WRN* expression in the prediction of chemosensitivity or radiosensitivity, we consider *WRN* expression status as a significant biomarker with the potential to be used as therapy target. In fact, both gastric cancer patients [42] and colorectal cancer patients [43] showed superior response to irinotecan in case of *WRN* hypermethylation. The administration of WRN-siRNA in a murine xenograft model of hypopharyngeal carcinoma inhibited tumor growth. The combination of siRNA with cisplatin further augmented the antitumor effect [41]. The inhibition of WRN might serve as specific therapy in WRN-dependent carcinomas in the future.

The main limitation of our study is the small numbers of samples and examined genes. The aim of this

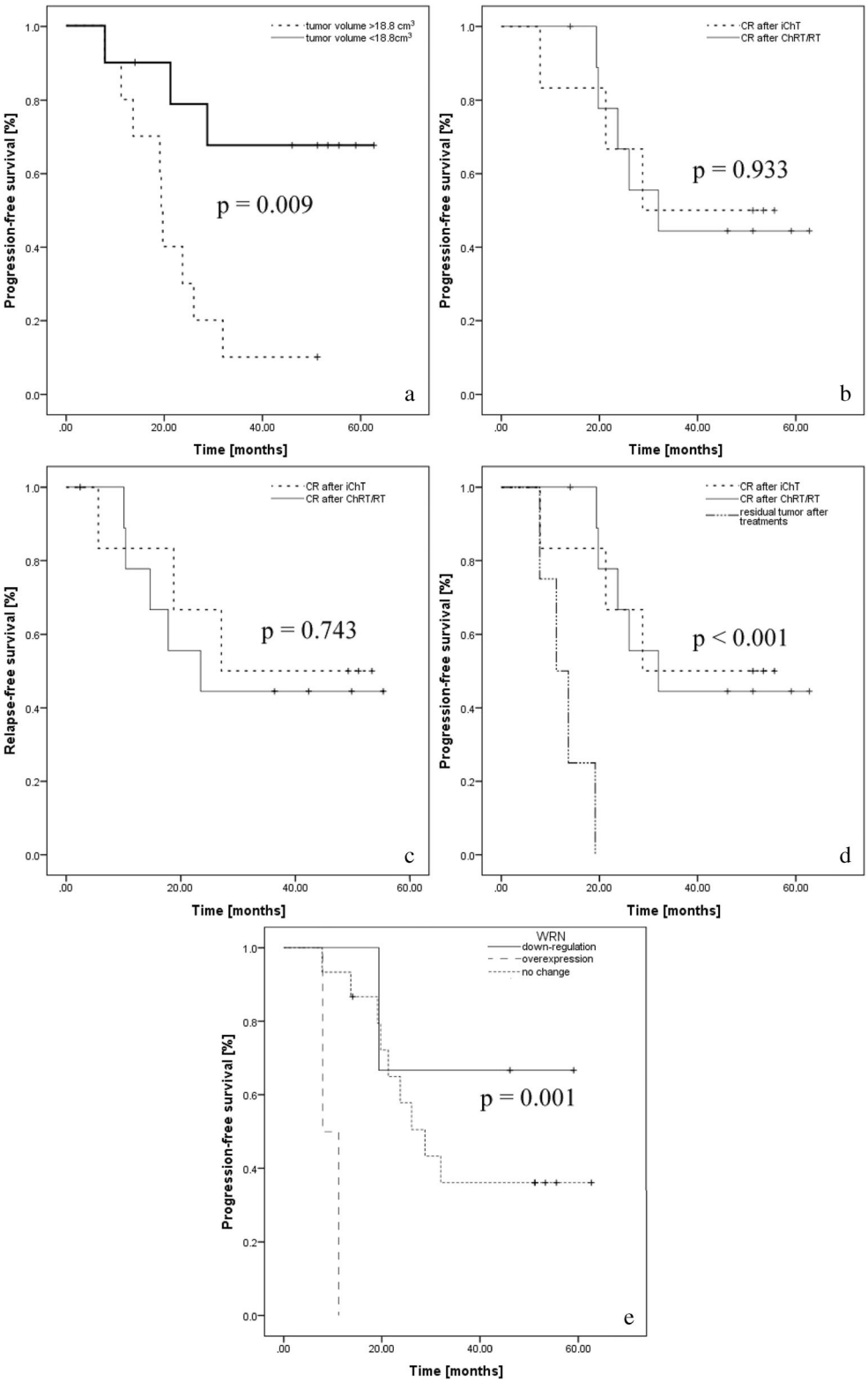

Fig. 5 Kaplan-Meier survival analysis. Relapse-free survival (RFS) was calculated in the patients with CR from the date of the diagnosis of being tumor-free to the date of tumor relapse, or the date of death, or the end of the follow-up. Progression-free survival (PFS) was calculated from the first day of the ChT to the date of any tumor progression (local, regional relapse or distant metastasis), the date of death, or the end of the follow-up. **a** PFS according to initial tumor volume (>18.8 cm^3 vs. ≤18.8 cm^3); **b** PFS and **c** RFS in the patients who achieved CR after iChT or after ChRT/RT. **d** PFS in the study population according to the response to therapy. The patients who achieved CR after iChT or after ChRT/RT (n = 16) versus those who never achieved CR (n = 4). **e** PFS in the overall patient population according to the WRN expression status. The p-values were calculated with the Log Rank (Mantel-Cox) test

pilot study was to identify genes involved in DNA repair processes which could be further tested in broader populations of patients.

In summary, our results suggest that *REV1* carry predictive information on sensitivity to ChRT/RT, while the expression of *WRN* was related to the patient outcome.

These biomarkers should be investigated further in larger study groups.

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