Original Article

Hypermethylation of *RAD51L3* and *XRCC2* Genes to Predict Late Toxicity in Chemoradiotherapy-Treated Cervical Cancer Patients

(DNA methylation / *RAD51* gene family / cervical cancer / late toxicity)

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Abstract. Cervical cancer affects women worldwide, especially in developing countries. Approximately 500,000 cases of this disease are diagnosed per year. The method of choice in the treatment of advanced cervical cancers (in accordance with the International Federation of Gynecology and Obstetrics staging system (FIGO) starting from stage IIB) is combined radiotherapy with concomitant chemotherapy. This treatment provides good tumour control, but it carries a risk of late complications in the irradiated area in 10–15 % of cases. Methylation is one of the methods of epigenetic control, which has an important role in gene expression. Aberrant methylation of normal CpG islands in promoters of tumour suppressor genes such as *RB, p53* or DNA reparation genes *ATM, BRCA1,2,* and *RAD51* gene family causes silencing of their function and cell cycle deregulation, which is one of the efficient ways of neoplastic transformation. The significantly decreased expression of molecules involved in DNA response may cause facilitated radiosensitivity in predisposed individuals. We looked for the relationship between hypermethylation of 18 DNA reparation genes and late toxicity occurrence in cervical cancer patients treated by chemoradiotherapy using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). The cut-off value for the hypermethylation was set at 10 %. We confirmed significant association between promoter hypermethylation in the *XRCC2* gene and occurrence of late grade III–IV toxicity in cervical cancer patients (P = 0.0357). This finding could be useful in the late toxicity prediction in radiotherapy-treated patients.

Introduction

Cervical cancer affects women worldwide, especially in developing countries. Approximately 500,000 cases of this disease are diagnosed per year and about 275,000 women die annually (Ellenson and Wu, 2004). In the Czech Republic the incidence of cervical cancer in 2009 was 19.2/100,000 inhabitants. The absolute number of new cases in this year was 1028 with 311 deaths.

The method of choice in the treatment of advanced cervical cancers (starting from FIGO stage IIB) is combined actinotherapy (external radiotherapy and brachytherapy) with concomitant chemotherapy. This treatment provides good disease control, but it carries a risk of late complications in the irradiated area. These complications affect 10–15 % of patients and increase morbidity and mortality in the post-treatment phase. Late effects of radiotherapy are first manifested at six months after the end of the treatment and mostly include tissue fibrosis, necrosis, and mucous membrane atrophy, which primarily affect the rectum, bladder, and small intestine. In the worst cases, these complications may lead to perforation of the intestine and bleeding, or formation of fistulae.

It has been shown that inactivation of tumour suppressor genes and activation of oncogenes play a significant role in carcinogenesis, caused by the genetic and epigenetic alterations. In the past, it was generally

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Abbreviations: DSB – double-strand break, FIGO – International Federation of Gynecology and Obstetrics staging system, HR – homologous recombination, ICRU – International Commission of Radiation Units and Measurements, MS-MLPA – methylation-specific multiplex ligation-dependent probe amplification, RTOG/EORTC – Radiation Therapy Oncology Group/European Organisation for Research and Treatment of Cancer.

thought that genetic mutation was a key event in tumour pathogenesis, especially somatic mutation of tumour suppressor genes. With deeper understanding of carcinogenesis in recent years, increasing evidence has shown that epigenetic silencing of these genes is a result of aberrant hypermethylation of CpG islands in promoters as well as of histone modification and is essential to carcinogenesis and metastasis. It is well established that cancer cells evolve as a result of deregulation in the normal cell cycle. Adequate regulation of the cell cycle is essential for all cell types and requires a great number of participant molecules. Methylation is one of the ways of epigenetic control, which plays an important role in gene expression. Aberrant methylation of normal CpG islands in promoters of tumour suppressor genes such as *RB, p53* or DNA reparation genes *ATM*, *BRCA1,2,* and *RAD51* gene family causes silencing of their function and cell cycle deregulation, which is one of the efficient ways of neoplastic transformation.

The *RAD51* gene family consists of several genes, which encode proteins with DNA-stimulated ATPase activity and property for preferential binding to single-stranded DNA and forming complexes with each other (Thaker, 2005). RAD51 participates in the common DNA damage response pathway associated with activation of homologous recombination (HR) and double-strand break (DSB) repair. In humans, RAD51 paralogues consisting of five proteins (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) facilitate HR mediated by protein RAD51 (Kawabata et.al., 2005). They are key components of HR, and their loss can result in developing extreme sensitivity to certain DNA-damaging agents and profound genetic instability (Sonoda et al., 1998). It has been shown that RAD51-like proteins interact in at least two complexes (Masson et al., 2001), with potential roles in both early and late HR processing (French et al. 2002; Liu et al., 2004), but their functions are otherwise poorly understood.

The *RAD51* gene family is one of the many studied gene groups associated with tissue radiosensitivity or tumour ionizing radiation responsiveness in cervical cancer. As reported, late toxicity is quite a severe and frequent complication in radiotherapy-treated patients and its prediction is at present impossible. Recently, there has been a huge progress in the field of molecular biology and many researchers have been using molecular biologic methods in the quest for genetic predictors of late radiation toxicity, radiosensitivity of normal tissues or tumour response to ionizing radiation in these cancers. For example, Zempolich et al. (2008) compared gene expression in pre-treatment and post-treatment (after chemo-irradiation) cervical cancer tissue. They established that after chemoradiotherapy, the expression ($P < 0.018$) of the genes that are components of the DNA damage response (*H2AX, RAD51, RAD53*) was significantly down-regulated. This significantly decreased expression may indicate that in some tumours, radiation sensitivity is facilitated by a diminished DNA repair response. Similar outcomes can be expected in normal tissues, while in predisposed individuals the radiosensitivity may be facilitated. These results are promising; however, no study is currently available that could be applied in general practice as a reliable late toxicity or tumour response predictive factor.

In our study, we looked for the relationship between hypermethylation of 18 DNA reparation genes and late toxicity occurrence in cervical cancer patients treated by chemoradiotherapy.

Material and Methods

Patients

We included 54 women in our study. The remaining 54 women were patients with advanced cervical cancer (FIGO stage IIB and higher) who received treatment at the Oncology and Radiotherapy Department, Faculty Hospital Hradec Králové in 2001–2010. At the time of publication, none of the patients had a sign of a disease recurrence. Principal demographic and clinical characteristics are described in Table 1.

The patients were enrolled in the study randomly, as they presented themselves for outpatient examination. Inclusion criteria were: cervical cancer diagnosis, clinical stage illness FIGO IIB and higher, chemoradiotherapy received, and follow-up six months or more. Before blood was taken, all patients signed an informed consent form for examination of DNA from peripheral blood and storage of DNA. The examination received approval of the ethics committee.

Treatment and follow-up

All patients received concomitant chemoradiotherapy, which included external radiotherapy of the pelvis delivered using the four-field BOX technique, which

Table 1. Demographic characteristics of the study population

Variables	N	Variables	N
Gender		**FIGO Stage**	
Female	54	IIB	26
Age (mean)		IIIB	28
Patients	47 years	**RT pelvis dose**	
		46/48.6 Gy	14
Follow-up (mean)	6 years	50/60 Gy	40
BMI (mean)	25	**RT parametrium dose**	
PS WHO		14 Gy	27
0	34	9 Gy	12
1	17	**RT PALU dose**	28
2	1	**BRT dose**	
3	2	18/24 Gy	40
Histopathology		28/30 Gy	14
SC-ca	52	**Chemotherapy**	54
Adenocarcinoma	2	cDDP	44
Grading		Taxol	10
1	8		
2	32		
3	15		

RT – radiotherapy; BRT – brachyradiotherapy; PALU – paraaortal lymph nodes; cDDP – cisplatin

Table 2. Genes in the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) KIT ME046 Repair genes (MRC Holland)

Gene	Name	Probes	Chromosomal location
ANKRD49	ankyrin repeat domain 49	08848-L08970	11q21
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1	08847-L08969	19q13.32
TGFBR1	transforming growth factor, β receptor 1	04644-L04028	9q22.33
PALB2	partner and localizer of BRCA2	10577-L12888	16p12.1
RPA2	replication protein A2	10572-L09319	1p35.3
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	08865-L12918	14q32.33
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	03975-L12955	9q21.33
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2	08863-L12919	7q36.1
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	08864-L09886	14q32.33
ESCO2	establishment of cohesion 1 homologue 2	10569-L12903	8p21.1
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2	08861-L08983	7q36.1
RAD51C	RAD51 homologue C	08852-L08974	17q22
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2	08862-L08984	7q36.1
MUS81	MUS81 endonuclease homologue	10573-L09315	11q13.1
ATR	ataxia telangiectasia and Rad3 related	08841-L09569	3q23
SYK	spleen tyrosine kinase	04521-L03974	9q22.2
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1	08846-L12920	19q13.32
ESCO2	establishment of cohesion 1 homologue 2	09153-L09311	8p21.1
RPA2	replication protein A2	09159-L09317	1p35.3
RAD51L3	RAD51 homologue D	08856-L08978	17q12
ATR	ataxia telangiectasia and Rad3 related	08842-L08964	3q23
RAD51C	RAD51 homologue C	08853-L08975	17q22
RAD51L3	RAD51 homologue D	08855-L08977	17q12
TOPBP1	topoisomerase (DNA) II binding protein 1	08860-L12921	3q22.1
APC	adenomatous polyposis coli	10363-L01968	5q22.2
EME1	essential meiotic endonuclease 1 homologue 1	08843-L08965	17q21.33
TOPBP1	topoisomerase (DNA) II binding protein 1	08859-L08981	3q22.1

was followed by a boost to the parametrium and, in indicated patients, also a boost to the paraaortic lymph nodes. During or after radiotherapy, patients received intrauterine brachytherapy with a Fletcher three-channel applicator (Varian Medical Systems, Inc., Palo Alto, CA); the radiation dose was applied to point A according to the International Commission of Radiation Units and Measurements (ICRU) 38.

Because in the past the treatment protocol had been changed, and due to the overall condition of certain patients in the course of the treatment, not all patients received the same dose of ionizing radiation during radiotherapy (including external radiotherapy and brachytherapy) and the same dose or type of chemotherapy. Treatment details are described in Table 1 mentioned above.

After the end of the treatment, the patients were monitored as outpatients every six months for a period of two years, after which they were monitored once a year. The patients underwent physical examination and imaging examination (X-ray of the thorax, ultrasound of the stomach and lesser pelvis). In case of complications the outpatient visits were made more frequently.

Late complications after radiotherapy were evaluated according to the Radiation Therapy Oncology Group/European Organisation for Research and Treatment of Cancer (RTOG/EORTC) guidelines. In patients with complications of grade I–II, the overwhelming majority suffered from proctitis and cystitis. The majority of patients with grade III–IV complications had ileus or rectovaginal or vesicovaginal fistulae, in some cases with perforation of the intestine. One patient had osteosclerosis with spontaneous fracture of the pelvic girdle. Patients were divided into three groups: group without complications, group with grade III–IV complications and group with grade I–IV complications. Thirteen pa-

tients were allocated to the group without complications, 25 to the group with grade III–IV complications and 42 patients to the group with any complications.

Data collection

Two test tubes of uncoagulated blood were collected from each patient. From 200 µl of blood, DNA was extracted using a micro-column (QIAamp Mini Blood Kit, Qiagen, Mississauga, ON, Canada).

Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA)

The present study used the MS-MLPA probe set ME046-A1 (MRC-Holland, Amsterdam, The Netherlands), which can simultaneously check for aberrant methylation in 18 tumour suppressor genes (Table 2). Probe sequences, gene loci and chromosome locations can be found at http://www.mlpa.com. Individual genes were evaluated by two probes, which recognized different *Hha*I restriction sites in their regions. The experimental procedure was carried out according to the manufacturer's instructions, with minor modifications.

In short, DNA (100 ng) was dissolved in up to 5 µl TE-buffer (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0), denatured and subsequently cooled down to 25 °C. After adding the probe mix, the probes were allowed to hybridize (overnight at 60 °C). Subsequently, the samples were divided into two halves: in one half, the samples were directly ligated, while in the other half ligation was combined with the *Hha*I digestion enzyme. This digestion resulted in ligation of the methylated sequences only. PCR was performed with all the samples using a standard thermal cycler (GeneAmp 9700, Applied Biosystems, Foster City, CA), with 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min with final extension of 20 min at 72 °C. Aliquots of 0.6 µl of the PCR reaction were combined with 0.2 µl LIZ-labelled internal size standard (Applied Biosystems) and 9.0 µl deionized formamide. After denaturation, fragments were separated and quantified by electrophoresis in an ABI 3130 capillary sequencer and analysed using GeneMapper4.0 (both Applied Biosystems). Peak identification and values corresponding to the peak size in base pairs (bp) and peak areas were used for further data processing. The methylation dosage ratio was obtained by the following calculation: Dm = (Px/Pctrl)Dig/ (Px/Pctrl)Undig, where Dm is the methylation dosage ratio, Px is the peak area of a given probe, Pctrl is the sum of the peak areas of all control probes, Dig stands for *Hha*I-digested sample and Undig for undigested sample. Dm can vary between 0 and 1.0 (corresponding to 0–100 % of methylated DNA). Based on previous experiments, we considered a promoter to show methylation if the methylation dosage ratio was ≥ 0.10, which corresponds to 10 % of methylated DNA (Nygren et al., 2005).

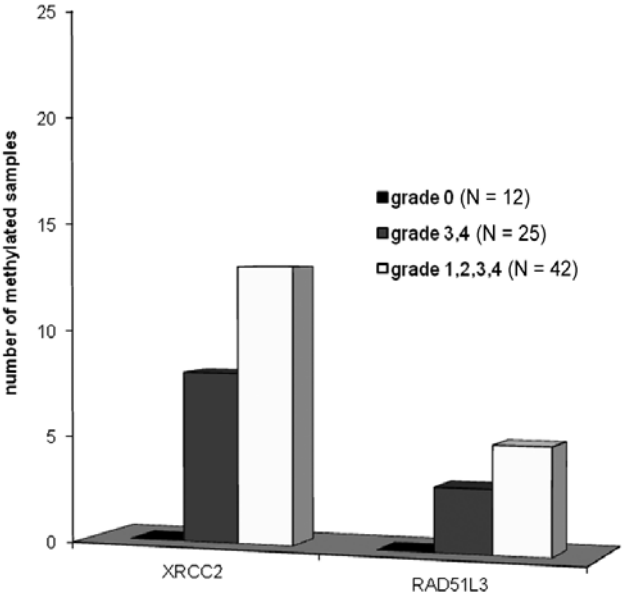

Fig. 1. Statistical comparison between the groups of patients using Fisher's exact test and P value < 0.05

CpG universal methylated and unmethylated DNA (Chemicon International, Temecula, CA) were used in every run as controls.

Statistical analysis

Proportions were compared by two-tailed Fisher's exact test. Associations with P value < 0.05 were considered to be significant (Fig. 1).

Results

In this study we tested the presence of gene hypermethylation in DNA samples from 54 cervical cancer patients using MS-MLPA. After that we searched for the association between gene hypermethylation and late post-irradiation toxicity. Using a 10% cut-off for methylation we observed statistically significantly higher methylation in the *XRCC2* gene (P = 0.0497) and non-significantly higher methylation in the *RAD51L3* gene (P = 0.34) in the group of patients with any complications than in the group without complications (Table 3).

Similar outcomes were obtained in comparing the group without complications with the group with grade III–IV complications. Using 10% cut-off there was significantly higher methylation in the *XRCC2* gene (P =

Table 3. Promoter gene hypermethylation using a 10% cut-off in subgroups of patients

Hypermethylated genes with 10% cut-off	Grade III–IV complications – N	Grade I–IV complications – N	Without complications – N
XRCC2	22	30	0
RAD51L3	3	12	0

0.0357) and non-significantly higher methylation in the *RAD51L3* gene (P = 0.5367) in the patient group with grade III–IV complications than in the group without complications.

For other tested genes from Table 2 the methylation rate did not exceed the 10% threshold.

Discussion

It is not known why some patients develop late radiation toxicity, and it is impossible to predict before treatment which patients will have long-term health problems after radiotherapy. The late toxicity risk can only be partly explained by clinical factors such as age, patient's condition, and radiation dose and volume. One theory is that some patients are genetically predisposed to developing severe late radiation toxicity. In the past decade, several research groups have tried to develop assays for predicting radiation toxicity in normal tissues (Turesson et al., 1996; Borgman et al., 2002, Thacker and Zdzienicka, 2004). However, the resulting data are contradictory and the outcomes have marginal significance.

In this study, we looked for markers of epigenetic predisposition for late radiation toxicity using the MS-MLPA method and our study is one of the first studies performed in cervical cancer patients. We discovered significant hypermethylation of the *XRCC2* gene and non-significant hypermethylation of the *RAD51L3* gene in radiosensitive cervical cancer patients with late complications after chemoradiotherapy. We assumed that this hypermethylation causes down-regulation of these genes and could be one of the causes of increased radiosensitivity, similarly as Zempolich et al. (2008) in their study. Another similar study of Kithara et al. (2002) established significant down-regulation of the *XRCC5* gene in a radiosensitive group of cervical cancer patients ($P < 0.05$). This gene is also involved in DSB repair, and deficiency of its gene product makes cells hypersensitive to ionizing radiation (Zhu et al., 1996). In our study we examined another 16 genes, but the percentage of their promoter methylation did not cross the 10% border. This could be caused by unsuitable gene selection or by a small number of the study cases.

Previous studies have reported that late and acute toxicity after radiotherapy is substantially determined by genetic predisposition, but the risk factors such as age, dose volume or comorbidities also play a role in acute or late toxicity development. However, no single gene or functionally related set of genes was found to be perfectly correlated with the observed clinical radiation toxicity (Svensson et al., 2006). Rieger et al. (2004) have suggested a link between acute toxicity and alterations in six main cellular processes: DNA repair, stress response, cell cycle, ubiquitination, apoptosis, and RNA processing. Acute and late toxicity are similar processes, and this finding supports the hypothesis that individual susceptibility to late radiation toxicity is substantially determined by genetic predisposition and is not associated with one particular gene.

In conclusion, we confirmed only one significant association between promoter hypermethylation in the *XRCC2* gene and occurrence of late toxicity in cervical cancer patients by using MS-MLPA. This hypermethylation causes silencing of the gene function and contributes to incorrect DNA reparation, which is one of the ways of late radiation tissue injury. Our results are in accordance with other similar studies, but additional, broader research is needed. The testing of *XRCC2* gene methylation, in the future, may by one of the methods of pretreatment radiosensitivity prediction and might help researchers to develop therapeutic interventions that would minimize the late radiation toxicity in vulnerable individuals.

Aknowledgement

The authors declare that there are no conflicts of interest.

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