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New specific molecular targets for radio-chemotherapy of rectal cancer

Kristin Snipstad^{a,1}, Christopher G. Fenton^{a,1}, Jørn Kjæve^b, Guanglin Cui^a,
Endre Anderssen^c, Ruth H. Paulssen^{a,*}

^aLaboratory of Molecular Medical Research, Institute of Clinical Medicine, University of Tromsø, N-9037 Tromsø, Norway

^bDepartment of Gastric Surgery, University Hospital of North-Norway, N-9038 Tromsø, Norway

^cInstitute of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology (NTNU), N-7006 Trondheim, Norway

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ABSTRACT

Patients with locally advanced rectal cancer often receive preoperative radio-chemotherapy (RCT). The mechanisms of tumour response to radiotherapy are not understood. The aim of this study was to identify the effects of RCT on gene expression in rectal tumour and normal rectal tissue. For that purpose tissue samples from 21 patients with resectable adenocarcinomas were collected for use in whole genome-microarray based gene expression analysis. A factorial experimental design allowed us to determine the effect of RCT on tumour tissue alone by removing the effect of radiation on normal tissue. This resulted in 1327 differentially expressed genes in tumour tissue with $p < 0.05$. In addition to known markers for radio-chemotherapy, a Gene Set Enrichment Analysis (GSEA) showed a significant enrichment in gene sets associated with cell adhesion and leukocyte transendothelial migration. The profound change of cell adhesion molecule expression in rectal tumour tissue could either increase the risk of metastasis, or decrease the tumour's invasive potential.

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1. Introduction

Over the past decade neoadjuvant radio-chemotherapy (RCT) has been increasingly employed in the treatment of rectal cancer. In Norway 10% of the patients with rectal cancer received RCT prior to surgery in 1995. Last year this number was increased to 40%. Clinical trials have shown a reduction in tumour size and stage (Bosset *et al.*, 2005), and a significantly reduced risk of local recurrence (Bosset *et al.*, 2006; Gérard *et al.*, 2006; reviewed in Glynne-Jones and Harrison, 2007; Rödel and Sauer, 2007; Wong *et al.*, 2007). Trials indicate that preoperative radiotherapy (PRT) does not increase postoperative mortality and that the acute and chronic toxicity of the treatment is acceptable (Kapiteijn *et al.*, 2001; Marijn

et al., 2002). *In vitro* and *in vivo* model systems have been able to give an insight as to how the tissue responds to radiation (for review see Rosen *et al.*, 1999). Cell types involved in the inflammatory reaction to tissue injury have been shown to be altered after short-term radiation (Nagtegaal *et al.*, 2002), and cellular responses are modulated by a diverse panel of integrin signalling cascades (Cordes and Meineke, 2004).

Studies comparing human rectal normal and cancer tissues using microarray technology are sparse. Information gathered about the molecular events triggered by irradiation has been previously investigated in attempts to predict the responses to both PRT and RCT (Ghadimi *et al.*, 2005; Watanabe *et al.*, 2006; Kim *et al.*, 2007; Ojima *et al.*, 2007). A study using tailored cancer microarrays showed that more than

* Corresponding author. Tel.: +47 776 45480.

E-mail address: ruthp@fagmed.uit.no (R.H. Paulssen).

¹ These authors contributed equally to this work.

Table 1 – Rectal cancer patient material characteristics. TNM-stage after MRI. APR, abdominoperineal resection; LAR, low anterior resection.

Variables	Non-irradiated	Irradiated
Gender (M/F)	6/5	8/2
Age, mean (range)	74.0 (52–85)	68.3 (55–82)
TNM-stage before treatment		
I	1	0
II	1	0
III	8	9
IV	1	1
Follow-up		
R 1–2 resection	2	0
Deceased	4	3
Non-cancer related death	1	2
Local recurrence	0	1
Distant metastasis	2	2
Surgery		
LAR	8	5
APR	3	5

90% of cancer-related genes remained unchanged upon radiation and that key genes involved in the control of apoptosis, proliferation and tissue repair were affected, while the effects on normal tissue appeared to be limited (Nagtegaal et al., 2005). The use of tailored cancer microarrays provides only a limited view of the effect of radiation on human tissue. Whole genome-based microarray might broaden the information collected about the molecular events induced by RCT. The aim of this study was to improve the understanding of the global transcriptional responses of RCT by comparing the effect of treatment on gene expression on both tumour and normal rectal tissue from Norwegian patients. This is to our knowledge the first whole genome study to investigate the effect of RCT on tumour rectal tissue samples.

2. Results

2.1. Patients

In this study, 21 patients with rectal cancer were included. Eleven patients had surgery only and ten patients received RCT preoperatively. The patients provided tissue samples of irradiated and non-irradiated tumour tissue and normal tissue for genome-wide gene expression analysis. Mean observation period was 40 months. TNM classification before treatment is shown in Table 1. For several patients in the non-RCT group neoadjuvant treatment was denied due to the general condition of the patient. Among the ten patients who received RCT, five had excellent responses with scattered tumour cells only in fibrotic tissue (response rated according to Mandard et al., 1994). In three patients tumours did not respond to RCT, while the remaining patients had tumours with intermediate responses.

2.2. Gene expression analysis

The molecular responses in tissue samples from rectal cancer patients receiving RCT were studied by measuring

Figure 1 – Comparison of treatment groups of rectal cancer patients. The Venn diagram shows the number of all differentially expressed genes across different comparisons: NR-NN, irradiated normal rectal tissue vs. non-irradiated normal rectal tissue; NR-NN, irradiated rectal tumour tissue vs. non-irradiated rectal tumour tissue; and comparison of NR-NN with TR-TN. The number of differential genes with $p < 0.05$ of each comparison is indicated, respectively.

genome-wide transcript-level changes using human genome survey microarrays representing 29,098 genes. Figure 1 shows the number and distribution of all 4616 differentially expressed genes with $p < 0.05$ across different comparisons in this study. Principal component analysis (PCA) (Figure 2A) revealed that the major variability in the data set is caused by the difference between irradiated and non-irradiated rectal tumour samples. To isolate any systematic trends within the normal samples, an additional PCA was run on the normal tissue samples only. To further highlight the difference between the groups a bridge partial least squares (PLS) model was used to extract a subspace where the group differences are more obvious than in PCA (Figure 2B). In order to aid in the verification of tumour/normal sample classification additional samples were taken from three patients. After data analysis using the LIMMA package (Smyth, 2004) the significance was determined at the 0.05 level corrected for false discovery rate using the method of Benjamini and Hochberg (1995). The application of a factorial experimental design revealed in a total of 1327 differentially expressed genes were found in the group of irradiated tumour samples, of which 646 genes were up- and 681 down-regulated (Supplemental list 1). Only 71 genes were affected in normal tissue, of which 39 were up-regulated and 32 down-regulated (Supplemental list 2). The two potential non-responders indicated in Figure 2B were excluded from the data set prior to further data analysis.

Figure 2 – Principal component analysis (PCA) and bridge partial least square analysis (PLS) on different rectal cancer patient samples. (A) Two-dimensional PCA of differentially expressed genes ($p < 0.05$), derived from 20 patients with rectal cancer, before and after radio-chemotherapy (RCT), showing separation of the different sample groups. (B) The plot depicts the scores of the PLS model. Components 1 and 2 (left) look similar to PCA analysis shown in (A), but component 3 (right) clearly separates the sample groups for normal rectal tissue. All samples are colour coded according to group. Black: NN (normal rectal tissue); green: NR (irradiated normal rectal tissue samples); blue: TN (rectal tumour tissue samples); red: TR (irradiated rectal tumour samples). *Potential non-responders.

2.3. Gene set enrichment analyses

Gene sets used in Gene Set Enrichment Analysis (GSEA) were derived from protein Analysis THrough Evolutionary Relationships (PANTHER; <http://www.panther.org/>). PANTHER biological processes, PANTHER molecular function, and KEGG pathways (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.jp/Kegg/>) are shown as BPXXXX, MFXXXX, and hsaXXXX, respectively in Table 2. GSEA analysis comparing tumour (TN) versus irradiated tumour (TR), showed a significant normalized enrichment score (NES), and a false discovery rate (FDR; q-value) in gene sets involved in cell-adhesion, cell adhesion molecules, leukocyte transendothelial migration, etc. Similar enrichments were not found for normal (NN) versus normal irradiated (NR) or normal (N) versus tumour (T) GSEA comparisons. The difference in cell adhesion gene expression between irradiated rectal tumour and non-irradiated rectal tumour, as well as normal irradiated and non-irradiated rectal tissue with genes $p < 0.001$ are shown in Figure 3 and Supplementary list 3. Here, only genes within the PANTHER BP00124 cell adhesion gene set (618 genes) and with two sided t-test p -value < 0.001 are shown.

2.4. Quantitative real-time PCR

Ten differentially expressed genes were validated by quantitative real-time PCR using TaqMan[®] Inventory assays as described in detail in Section 5: Insulin-like growth factor 1

(IGF1), angiopoietin 1 (ANGPT1), prostaglandin E receptor 3 (PTGER3), dermatopontin (DPT), *v-myb* myeloblastosis viral oncogene homologue (MYB), fatty acid binding protein 6 (FABP6), collagen type VI alpha 1 (COL6A1), matrix metalloproteinase 2 (MMP2), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and protocadherin 1 (PCDH1). The relative expression is shown in Table 3. Six genes (IGF1, ANGPT1, PTGER3, DPT, MYB, and FABP6) were tested on pooled samples, with five patients in each pool, as indicated. Four genes (COL6A1, MMP2, CEACAM1, and PCDH1) which are related to cell adhesion were tested on five irradiated tumour samples and five non-irradiated tumour samples. To see if there was a difference in expression related to tumour stage, we used patient tumour material representing different TNM stages. The results indicate that PCDH1 is up-regulated at TNM stages I and II, but down-regulated at TNM stages III and IV. The average relative expression as well as the fold-change calculated after microarray showed a down-regulation of PCDH1. Two RCT-treated stage III tumours showed fairly different relative expression of COL6A1 and resulted in a down-regulation of the gene at this TNM stage. Even though the level of expression varied, there was no change observed regarding up- and down-regulation for any genes or TNM stages tested.

2.5. Immunohistochemistry

Three differentially expressed genes that are involved in cell adhesion have been further validated on protein level by

Table 2 – Gene sets involved in cell adhesion. Comparisons of gene sets (GS) for cell adhesion used in Gene Set Enrichment analysis (GSEA) are indicated as rectal tumour tissue (TN), irradiated rectal tumour tissue (TR), normal rectal tissue (NN), and irradiated normal rectal tissue (NR). The FDRs (q-value) are set to ≤ 0.05 . The size indicates the number of genes representing the gene set, BPXXXXX and MFXXXXX indicate biological processes and molecular functions obtained with PANTHER, and hsaXXXXX indicates pathways obtained with KEGG.

GS	Size	Source	TN vs. TR		NN vs. NR		NN, NR vs. TN, TR	
			NES	FDR	NES	FDR	NES	FDR
BP00107	253	Cytokine and chemokine mediated signalling pathway	−1.59	0.10	−1.05	0.67	−0.84	0.76
BP00120	408	Cell adhesion mediated signalling	−1.73	0.08	−0.99	0.73	−0.86	0.76
BP00124	618	Cell adhesion	−1.76	0.10	−1.29	0.49	−1.00	0.58
BP00275	69	Extracellular matrix protein mediated signalling	−2.01	0.01	−1.73	0.31	−1.31	0.27
BP00287	391	Cell motility	−1.75	0.08	−1.39	0.39	−1.11	0.48
MF00040	103	Cell adhesion molecule	−1.98	0.02	−1.48	0.56	−1.10	0.47
MF00174	50	Complement component	−1.71	0.09	−1.03	0.80	−1.14	0.45
MF00179	86	Extracellular matrix structural protein	−1.87	0.05	−1.51	0.53	−1.33	0.29
MF00180	98	Extracellular matrix glycoprotein	−1.97	0.02	−1.47	0.05	−1.24	0.40
MF00219	76	Annexin	−1.77	0.06	−1.48	0.52	1.13	0.45
MF00255	74	Non-motor microtubule binding protein	−1.78	0.06	−1.02	0.79	−1.40	0.24
MF00255	99	Actin binding cytoskeletal protein	−1.81	0.04	−1.26	0.53	−0.76	0.83
MF00269	46	SNARE protein	−1.84	0.04	−1.39	0.47	−0.92	0.68
hsa04060	311	Cytokine–cytokine receptor interaction	−1.67	0.06	−1.11	0.65	−1.18	0.48
hsa04510	195	Focal adhesion	−1.99	0.03	−1.94	0.08	−1.09	0.53
hsa04512	438	ECM receptor interaction	−1.63	0.08	−1.57	0.42	−1.26	0.41
hsa04514	353	Cell adhesion molecules (CAMs)	−1.94	0.01	−1.10	0.66	1.05	0.48
hsa04610	68	Complement and coagulation cascades	−1.99	0.02	−1.46	0.44	−1.38	0.32
hsa04670	175	Leukocyte transendothelial migration	−1.93	0.02	−1.38	0.58	1.10	0.44
hsa04810	235	Regulation of actin cytoskeleton	−1.70	0.05	−1.24	0.60	1.15	0.42

immunohistological staining of paraffin-embedded rectal tissue samples. Figure 6 shows representative samples out of ten patients tested. Immunoreactivity of phospho-Histone H2A.X was not observed in the tumour-free adjacent mucosa before radiotherapy of normal rectal tissue (Figure 6A), but a few positive cells could be found after treatment (Figure 6B). Phospho-Histone H2A.X was only observed at a low density in the nuclei of cancer cells before RCT treatment (Figure 6C), but it was significantly increased in cancer cells after treatment (Figure 6D). In tumour-free adjacent mucosa, immunoreactive CD31 was found in both epithelium and stromal cells (Figure 6E). These findings were not influenced by treatment (Figure 6F), but RCT increased CD31 levels in cancer tissue (Figure 6G and H). The immunoreactivity of Collagen VI was diffusely distributed in the tumour stroma and slightly increased in cancer tissue after RCT treatment (Figure 6K and L for cancer, Figure 6I and J for control). LAMA4 immunoreactivity was slightly increased after RCT in cancer tissue (Figure 6M and N for control, Figure 6O and P for cancer).

3. Discussion

This study highlights the effect of RCT on gene expression profiles in both tumour and normal tissue from rectum, obtained with whole genome-based microarrays. Our data clearly show that RCT affects tumours to a greater extent

than normal tissue. A factorial experimental design allowed us to determine the effect of RCT on tumour tissue alone by removing the effect of radiation on normal tissue. It is hereby noted that the effects on tumour tissue are caused by the combination therapy with radiation and capecitabine. Since this is a standard of patient care it is not possible to look at radiation effects on tumour tissue per se.

3.1. RCT effect on rectal tumour tissue

GSEA revealed that several biological pathways profoundly affected by RCT are involved in cell adhesion (Table 2). Figure 4 shows some of the affected genes in two different KEGG pathways. Recent *in vivo* and *in vitro* studies, and some additional clinical settings, have described the effect of radiation on cell adhesion molecules that are involved in trans-endothelial migration and inflammation (reviewed by Baluna et al., 2006). Nagtegaal and co-workers showed that radiation of rectal tumour tissue affects genes that are involved in biological processes for cytokines and their receptors, metalloproteases and adhesion molecules (Nagtegaal et al., 2005). The amount of genes in these groups was limited, and only a few of these genes matched our results. Another study on patients with colorectal cancer who underwent PRT has shown that eight of the 20 up-regulated genes in PRT responders were linked to cell adhesion (Watanabe et al., 2006). The same genes are up-regulated in our study. However, studies of the effect of

Figure 3 – Genes involved in cell adhesion are specifically affected by RCT in rectal cancer tissue samples. The difference in the cell adhesion gene expression between radiated rectal tumour (TR, red) and rectal tumour (TN, green), normal rectal tissue (NN, black) and radiated normal rectal tissue (NR, blue) is shown. Gene expression values are shown in a spectrum where down-regulated is green, no change is black, and up-regulated is red. Only genes (47) within the PANTHER BP00124 cell adhesion gene set (618 genes) and with two-sided *t*-test $p < 0.001$ (TR vs. TN, NN, NR) are shown in the figure. Top panel of the heat map depicts patient sample groups. Black, non-radiated normal rectal tissue; blue, radiated normal rectal tissue; green, non-radiated rectal tumour tissue; red, radiated tumour rectal tissue.

irradiation on cell adhesion molecules have led to contradicting interpretations. Cell adhesion signalling mediates cell-ECM and cell-cell adhesion, and a deterioration of this adhesion is often observed in tumour cells potentially leading to enhanced metastasis (Camphausen et al., 2001; Wild-Bode et al., 2001; Rudmik and Maliocco, 2005). Or perhaps the reduction in cell adhesion is a result of fewer migrating tumour cells (Akimoto et al., 1998). Alternatively, changes in the tissue microenvironment and stromal-epithelial interactions, including for example remodelling of the ECM composition, have been reported (Barcellos-Hoff et al., 2005). In this study, the extracellular matrix proteins COL6A1 and LAMA4 have been found to be up-regulated in tumour tissue after RCT. It has been recently reported that increased expression levels of LAMA4 and COL6A1 are correlated with tumour progression and are proposed to be a new markers for tumour invasion and metastasis (Fujita et al., 2008; Huang et al., 2008).

As part of the immune response following PRT, integrins may serve as critical membrane receptors on diverse normal cells such as leukocytes, platelets or fibroblasts (Cordes and Meineke, 2005). Cell-matrix interactions are in part mediated through the β_1 -integrin pathway regulating cell survival, proliferation, adhesion and migration. We found that β_1 -integrin,

integrin-linked kinase (ILK) and serum response factor (SFR) are up-regulated in rectal tumour tissue after RCT. This is consistent with a report of radiation effects on COLO-320 cells, thereby emphasizing the role of this integrin in the adhesion to fibronectin and collagen (Meineke et al., 2002). Increased expression of β_1 -integrin and ILK might enable the cells to adhere to the endothelium, which might represent a prerequisite for metastasis (Cordes et al., 2002) and might lead to increased metastatic activity (Yasoshima et al., 1996; Okazaki et al., 1998). On the other hand, cells could also adhere more strongly in their environment by increasing functional surface receptor density, thereby preventing metastasis (Cordes et al., 2002). Increased expression of integrin $\alpha_5\beta_1$ has been associated with a loss of metastatic potential (Schirner et al., 1998). ILK interacts with the cytoplasmic domain of β_1 and β_3 integrins, and transmits signals from the extracellular matrix to actin filaments. Increased ILK expression has been associated with cellular differentiation in high turnover tissues but not generally with a malignant phenotype, thus making ILK an indicator of differentiation (Haase et al., 2008). Two of the down-regulated genes found in this study are E-cadherin (CDH1) and α_6 -integrin. Decreased expression of α_6 -integrin, together with an increased expression of β_1 -

Table 3 – Validation of microarray data by quantitative PCR. Total RNA samples were subjected to qPCR as described in detail in Section 5. A: For quantification pooled RNA (four pools with $n = 5$, representing irradiated and non-irradiated tumour samples) or single RNA samples ($n = 10$) were used, as indicated. Values are depicted as fold-change $\pm$ SD (microarray) and relative expression $\pm$ SD (qPCR). B: The relative expression of four genes from RNA of single tumour samples representing different TMN stages was determined. Values are depicted as relative expression $\pm$ SD.

A

Gene symbol	Fold-change	Relative expression
	(microarray)	(qPCR)
IGF1 ^a	↑ 2.73 ± 0.69	↑ 14.22 ± 4.36
ANGPT1 ^a	↑ 2.38 ± 0.59	↑ 9.60 ± 4.99
PTGER3 ^a	↑ 2.39 ± 0.19	↑ 25.43 ± 17.80
DPT ^a	↑ 4.26 ± 1.02	↑ 103.84 ± 19.60
MYB ^a	↓ −3.17 ± 0.32	↓ 0.11 ± 0.08
FABP6 ^a	↓ −3.26 ± 0.84	↓ 0.02 ± 0.05
COL6A1	↑ 2.22 ± 0.47	↑ 13.91 ± 15.99
MMP2	↑ 2.56 ± 0.58	↑ 23.16 ± 11.38
CEACAM1	↓ −3.06 ± 0.02	↓ 0.08 ± 0.05
PCDH1	↓ −1.09 ± 0.45	↓ 0.67 ± 0.24

B

Gene symbol	TNM Stage I (2)	TNM Stage II (2)	TNM Stage III (4)	TNM Stage IV (2)
COL6A1	↑ 10.14 ± 7.05	↑ 18.28 ± 11.30	↓ 0.77 ± 0.76	↑ 2275.55 ± 4.20
MMP2	↑ 13.56 ± 1.28	↑ 27.70 ± 0.99	↑ 10.35 ± 6.14	↑ 78.90 ± 11.13
CEACAM1	↓ 0.06 ± 0.01	↓ 0.93 ± 0.37	↓ 0.04 ± 0.01	↓ 0.02 ± 0.001
PCDH1	↑ 1.18 ± 0.05	↑ 2.72 ± 0.18	↓ 0.17 ± 0.04	↓ 0.65 ± 0.28

↑, up-regulated, ↓, down-regulated; SD, standard deviation; number of patients.
a pooled samples.

integrin is associated with malignant progression (Park et al., 2003), and loss of E-cadherin could increase the tumour's invasive potential (Vleminckx et al., 1991; Yasoshima et al., 1996; Luo et al., 1999; Ikeguchi et al., 2000). In addition to the decreased expression of E-cadherin, increased levels of SFR may play a role in the enhancement of cell motility and the invasiveness in colorectal cancer metastasis (Choi et al., 2009). The observed down-regulation of E-cadherin in our study is inconsistent with reports showing either up-regulation or unchanged expression levels for E-cadherin after irradiation (Ebara et al., 1998; Hardy et al., 2002; Nagtegaal et al., 2005).

Differential expressed genes in irradiated rectal cancer tissue are involved in leukocyte transendothelial migration (Figure 5). For instance, the chemokine and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), known to facilitate the binding of integrins to vascular cell adhesion molecules (VCAM) members (DeLisser et al., 1997), was up-regulated in tumour tissue after RCT and might reflect the response of the endothelial cells to injury by this treatment. In addition, both vascular cell adhesion molecule 1 (VCAM-1) and E-selectin (SELE) were up-regulated in tumour tissue after RCT. This is consistent with previous findings (Heckmann et al., 1998; Hildebrandt et al., 2002; Zempolich et al., 2008). Leukocyte adhesion and accumulation in tumour tissue is correlated with the extent of tumour cell death (Ryschic et al., 2003), and tumours infiltrated by lymphocytes have been shown to have a better prognosis (Emile et al., 2000; Gervaz et al., 2000; Okano et al., 2003). However, these findings are not conclusive, since another study showed that leukocytes may instead contribute to tumour progression (De Visser et al., 2006). Leukocyte

trafficking across the vessel wall is tightly regulated, and requires a coordinated expression of cell adhesion molecules on both activated endothelial cells and leukocytes, in addition to immobilization of chemokines on the epithelial surface (reviewed in van Buul and Hordijk, 2004). The pathway cascade in leukocyte trans-endothelial migration could be relevant for the adherence of circulating tumour cells and might resemble tumour metastasis. E-selectin has for instance been implicated to play a role in colon cancer metastasis (reviewed in Krause and Turner, 1999), and may increase tumour cell adhesion (Nübel et al., 2004). In addition, there are reports showing that some tumour cells use chemokines to spread through the body (Müller et al., 2001). One example is the chemokine receptor CXCR4 and its ligand CXCL12, both significantly up-regulated in our study. Increased expression of CXCR4 has been suggested to be a risk factor for the development of liver metastasis (Kim et al., 2005).

Tumour suppressor genes like transforming growth factor β (TGF- β) and its signal transducers Smad2 and Smad4 are up-regulated in our study, which is consistent with reports showing that activation of TGF- β is an early and sensitive response to irradiation (Barcellos-Hoff et al., 1994). TGF- β /Smad signalling might repress the expression of the oncogenic transcription factor c-Myc (Pietenpol et al., 1990), which is down-regulated in our study. In addition to acting as a tumour suppressor, TGF- β has also been shown to have a pro-tumorigenic effect (reviewed by Pardali and Moustakas, 2007). Induction of Ras could decrease the growth-inhibitory response to TGF- β (Fujimoto et al., 2001), and there are reports showing that K-ras signalling could play a role in the conversion of TGF- β from a tumour suppressor to a tumour

Only a few differentially expressed genes were observed in normal tissue and the expression profile is different found for irradiated tumour tissue. Several of the up-regulated genes are involved in signal transduction processes, and more

Figure 5 – Leukocyte transendothelial migration pathway is affected by RCT. The pathway for leukocyte transendothelial migration generated with KEGG is shown. Each bar (blue or red) represents a gene. Red bars indicate an increase in irradiated rectal tumour gene expression as compared to non-irradiated rectal tumour samples. Blue bars indicate a decrease. Bar height is inversely related to p -value; a larger bar represents a smaller p -value up to a maximum height at p -value less than 10^{-8} .

specifically to cell communication, cell surface receptor mediated signal transduction and intracellular signalling cascade (mostly the JNK cascade). The up-regulated genes FBJ murine osteosarcoma viral oncogene homologue B (FOSB), nuclear receptor subfamily 4, group A, member 1 (NR4A1) and nuclear receptor subfamily 4, group A, member 2 (NR4A2) are all associated with the transcription factor serum response factor (SFR), and may play a crucial role in tissue injury and ulcer healing (Chai and Tarnawski, 2002). Genes involved in pro-apoptotic signalling, like activating transcription factor 3 (ATF3), early growth response gene 1 (EGR1) and *v-fos* FBJ murine osteosarcoma viral oncogene homologue (FOS), showed increased expression. ATF3 is known to be induced by external stimuli such as IR and UV (Fan et al., 2002). EGR1 has been implicated to be central for tumour growth (Thiel and Cibelli, 2002), and may also increase the expression of ATF3 (Yamaguchi et al., 2006). Increased expression of dual specificity phosphatase 1 (DUSP1) was observed in irradiated rectal tumour tissue. DUSP1 is known to be an important partner in a negative feedback-loop of mitogen-activated protein kinase 3 (MAPK3) and mitogen-activated protein kinase 1 (MAPK1) (Lin and Yang, 2006; Owens and Keyse, 2007). DUSP1 has also been reported

to be involved in the regulation of immune function and cellular inflammatory responses (Wong et al., 2005). The up-regulated tumour suppressor gene *ras* homologue gene family member B (RHOB), which is required for p21 transcription, is suggested to play a role in a DNA-damage checkpoint, and may be linked to the actin-microtubule organization or vesicle transport (Prendergast, 2001).

4. Conclusions

RCT predominantly affects rectal tumour tissue in a specific manner. Genes that are associated with cell adhesion and leukocyte transendothelial migration are affected to a large extent. Most of the genes represented in the two pathways have not previously been reported to be affected by RCT. Therefore, these genes may represent new specific therapeutic targets in rectal cancer therapy. The observed changes in gene expression could either increase the risk of metastasis, or decrease the tumours invasive potential. Further investigations are needed to resolve the affect of RCT on tumour metastasis.

Figure 6 – Immunohistological staining of different rectal tissue samples. The immunoreactivity of phospho-Histone H2A.X (C, D) and CD31 (G, H) was significantly increased in cancer tissue after receiving RCT compared to untreated samples. The immunoreactivity of COL6A1 (K, L) and LAMA4 (O, P) was slightly increased in cancer patients after RCT. There were no changes observed for the expression pattern of these proteins in tumour-free adjacent mucosa (E, F: CD31; I, J: COL6A1; M, N: LAMA4) except for phospho-Histone H2A.X, which was increased (A, B). The slides were counterstained with haematoxylin, and the original magnification for images was 400×.

5. Experimental procedures

5.1. Patient material

The study group consisted of 21 patients with resectable adenocarcinoma of the rectum. Eleven patients had surgery only. The remaining ten patients received preoperative radio-chemotherapy (RCT). Each patient received 50 Gy (delivered as fractions of 2 Gy 25 times) during 5 weeks. In addition, patients received Capecitabine (Xeloda®, Roche) 825 mg/m², two times daily during the whole radiation period. Resection of the rectum was performed 4–6 weeks after RCT was ended. Tissue specimens were taken from both normal and tumour tissue of the rectum in both treatment groups. Tissue samples were kept in RNA storage buffer (RNA stabilization reagent, Qiagen GMBH, Germany) and stored at –70 °C prior to downstream analyses. The different TMN stages of tumours before treatment were assessed by magnetic resonance imaging (MRI) (Table 1). A written consent was obtained from all patients included in the study prior to the collection of specimens. The study protocol was approved by the Regional Ethics Committee of North-Norway (REK).

5.2. RNA preparation and quality/quantity control

Disruption and homogenization of tissue specimens were performed in lysis buffer using the MagNa Lyser Instrument (Roche Applied Science, Germany) according to the manufacturer's protocol. Subsequently, total RNA was isolated with the MagNa Pure Compact Instrument and the MagNa Pure Compact RNA Isolation Kit (Roche Applied Science, Germany) as previously described (Paulssen et al., 2006). RNA was quantified by measuring absorbance at 260 nm, and RNA purity was determined by the ratios OD_{260nm/280nm} and OD_{230/280nm} using the NanoDrop instrument (NanoDrop® ND-1000, Wilmington, USA). The RNA integrity was determined by electrophoresis using the Bio-Rad Experion Bioanalyzer (data not shown). All the RNA preparations were verified for possible genomic DNA contamination by a minus-RT-PCR conducted directly on RNA samples, with human genomic DNA as a positive control and amplification of the human housekeeping gene cyclophilin A. Genomic DNA was not detected in RNA preparations (data not shown).

5.3. Probe generation and array hybridization

Total RNA samples were processed into digoxigenin (DIG) - labelled cRNA using the Applied Biosystems Chemiluminescent

NanoAmp™ RT-IVT Labelling Kit (Applied Biosystems Inc., USA). Ten micrograms of labelled DIG-cRNA was injected into each microarray hybridization chamber, hybridized at 55 °C for 16 h, and visualized using the Applied Biosystems Chemiluminescence Detection Kit. The signals were detected by the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer. The Human Genome Survey Microarray v.2.0 (Applied Biosystems Inc., USA) with 32,878 probes for the interrogation of 29,098 genes was used for microarray analysis.

5.4. Data analysis and statistical analysis

The features were extracted from the arrays using the ABI 1700 software. Normalization was carried out using quantile normalization (Bolstad et al., 2003). For the purpose of finding differentially expressed genes, we applied an empirical Bayes analysis using the LIMMA package (Smyth, 2004), and significance was determined at the 0.05 level corrected for false discovery rate (FDR) using the Benjamini–Hochberg method (Benjamini and Hochberg, 1995). Principal component analysis (PCA) and partial least squares (PLS) were carried out on the data in order to visualize the data structure and look for potential outlier samples (Gidskehaug et al., 2004). GSEA was performed using the R statistical package (<http://www.broad.mit.edu/gsea/>).

5.5. Database submission of microarray data

The microarray data were prepared according to minimum information about a microarray experiment (MIAME) recommendations and deposited in the GENE Expression Omnibus (GEO) database: <http://www.ncbi.nlm.nih.gov/geo/>. The GEO accession number for the series is GSE15781.

5.6. Validation of microarray results by real-time PCR (qPCR)

Total RNA from four pools (two from irradiated tumour samples and two from non-irradiated tumours), each pool consisting of five samples, was used to test six genes associated with colon cancer (IGF1, ANGPT1, PTGER3, DPT, MYB, and FABP6). In addition, five non-pooled samples from each sample group (irradiated and non-irradiated tumours), representing different TNM stages was used particularly to test four genes involved in cell adhesion (COL6A1, MMP2, CEACAM1, and PCDH1). The total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Part Number 438814) and as described by the manufacturer's protocol. TaqMan PCR amplification was performed with an ABI HT7900 Fast Instrument using the TaqMan Gene Expression Assays (Applied Biosystems, USA). Cyclophilin A (PPIA) was used as reference gene. The following ABI Assay IDs can be found at www.appliedbiosystems.com: Hs00153126_m1, insulin-like growth factor (IGF1); Hs00181613_m1, angiopoietin 1 (ANGPT1); Hs00168755_m1, prostaglandin E receptor 3 (PTGER3); Hs00170030_m1, dermatopontin (DPT); Hs00193527_m1, v-myb myeloblastosis viral oncogene homologue (MYB); Hs00155029_m1, fatty acid binding protein 6 (FABP6); Hs00242448_m1, collagen type VI alpha 1 (COL6A1); Hs00234422_m1, matrix metalloproteinase 2 (MMP2);

Hs00236077_m1, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1); Hs00260937_m1, protocadherin 1 (PCDH1). Samples were run in triplicate and averaged for final quantification. The relative expression was calculated as previously described (Livak and Schmittgen, 2001), and processed by the Relative Expression Software Tool (REST) (Pfaffl et al., 2002).

5.7. Immunohistochemistry

Immunohistochemistry (IHC) was performed with a Vectastain ABC KIT (Vector Laboratories, Inc, Burlingame, CA, USA) according to the manufacturer's instructions and our published protocol (Cui et al., 2009). Antigen retrieval was achieved using EDTA buffer (pH 8.0) through microwave processing for phospho-Histone H2A.X and CD31 IHCs, proteinase K digestion for Collagen VI IHC, and Pepsin digestion for LAMA4 IHC, respectively. The sections were incubated overnight at 4 °C with anti-phospho-Histone H2A. X (Cell Signalling Technology Inc., Danvers, MA, USA), anti-CD31 (Abcam, Cambridge, UK), anti-Collagen VI (Abcam, Cambridge, UK) and anti-LAMA4 (Abcam, Cambridge, UK) individually. 3-Amino-9-ethylcarbazole (AEC, Vector Laboratories, Burlingame, CA, USA) was used as chromogen and Mayer's haematoxylin as counterstain. Routine negative control slides were used: (1) primary antibodies were substituted with the isotype-matched control antibodies; (2) secondary antibody was substituted with phosphate-buffered saline (PBS).

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Supplementary information

Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.molonc.2009.11.002.

Appendix A. Supplemental material

Supplementary information for this manuscript can be downloaded at doi: 10.1016/j.molonc.2009.11.002.

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