Early dynamic transcriptomic changes during preoperative radiotherapy in patients with rectal cancer: A feasibility study

Stephane Supiot, Wilfried Gouraud, Loïc Campion, Pascal Jezéquel, Bruno Buecher, Josiane Charrier, Marie-Francoise Heymann, Marc-Andre Mahé, Emmanuel Rio, Michel Chérel

AIM: To develop novel biomarkers of rectal radiotherapy, we measured gene expression profiles on biopsies taken before and during preoperative radiotherapy.

METHODS: Six patients presenting with a locally advanced rectal cancer (T>T2, N0/Nx, M0) eligible for preoperative radiotherapy (45 Gy in 25 fractions) were selected in a pilot study. Six tumor and 3 normal tissues biopsies were taken before and during radiotherapy, after a dose of 7.2 Gy at a median time of 1 h following irradiation (0:27-2:12). Tumor or normal tissue purity was assessed by a pathologist prior to RNA extraction. Mean RNA content was 23 µg/biopsy (14-37) before radiotherapy and 22.7 µg/biopsy (12-35) during radiotherapy. After RNA amplification, biopsies were analysed with 54K HG-U133A Plus 2.0 Affymetrix expression micro-arrays. Data were normalized according to MAS5 algorithm. A gene expression ratio was calculated as: (gene expression during radiotherapy - gene expression before radiotherapy)/gene expression before radiotherapy. Were selected genes that showed a ratio higher than ± 0.5 in all 6 patients.

RESULTS: Microarray analysis showed that preoperative radiotherapy significantly up-regulated 31 genes and down-regulated 6 genes. According to the Gene Ontology project classification, these genes are involved in protein metabolism (ADAMDEC1; AKAP7; CAPN5; CLICS; CPE; CREB3L1; NEDD4L; RAB27A), ion transport (AKAP7; ATP2A3; CCL28; CLICS; F2RL2; NEDD4L; SLC6A8), transcription (AKAP7; CREB3L1; ISX; PABPC1L; TXNIP), signal transduction (CAPN5; F2RL2; RAB27A; TNFRSF11A), cell adhesion (ADAMDEC1; PXDN; SPON1; S100A2), immune response (CCL28; PXDN; TNFRSF11A) and apoptosis (ITM2C; PDCD4; PVT1). Up-regulation of 3 genes (CCL28; CLICS; PDCD4) was detected by 2 different probes and up-regulation of 2 genes (RAB27A; TXNIP) by 3 probes.

CONCLUSION: Micro-arrays can efficiently assess early transcriptomic changes during preoperative radiotherapy for rectal cancer, and may help better understand tumor radioresistance.

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Key words: CCL28; CLICS; PDCD4; RAB27A; TXNIP; Protein metabolism; Cell adhesion; Cell migration; SPON1; Carboxypeptidase E
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Core tip: To develop novel biomarkers of radiotherapy for rectal cancer, we measured gene expression profiles on biopsies taken before and during preoperative radiotherapy in a pilot study. Microarray analysis showed that preoperative radiotherapy significantly up-regulated 31 genes and down-regulated 6 genes, involved in protein metabolism, ion transport, transcription, signal transduction, cell adhesion, immune response and apoptosis. Micro-arrays could efficiently assess early transcriptomic changes during preoperative radiotherapy for rectal cancer. This may help better understand tumor radioresistance.

INTRODUCTION

In patients with rectal adenocarcinoma, preoperative radiotherapy (RT), either alone or combined with chemotherapy, reduces the 5-year rate of local recurrence by 5% to 10%[1]. However, owing to high inter-individual variation, 6-18 patients have to be treated in order to avoid one recurrence. Identifying patients likely to benefit is thus essential.

End outcomes after preoperative RT can hardly be predicted by analyzing the expression of known proteins on pre-treatment biopsies[2] or clinical parameters[3]. However, micro-array gene expression profiling can help define diagnostic, prognostic and predictive factors for response to RT[4]. Transcriptomic profiles obtained on pre-RT biopsies can be used to identify patients with rectal adenocarcinoma who are likely to relapse despite appropriate treatment[5]. Validation studies on larger cohorts are ongoing.

Sequential biopsies have highlighted changes in tumor dynamics (proliferation, cell cycle, apoptosis) during pelvic RT and identified treatment targets that might modify RT outcomes in patients with cervical cancer, where tumor access is easier than for the rectum[6]. Although sequential biopsies have also revealed histological changes in rectal mucosa due to radiation toxicity, data are few[7,8]. Very recently, biopsy specimens could be obtained 7 d after starting chemoradiotherapy and provided interesting biomarkers of response to treatment in rectal cancer patients[9].

A study of radiation-induced cellular and biochemical changes in rectal tumors might help better understand radiation-induced cell death and identify new targets for enhancing RT efficacy. We postulated that sequential biopsies could be used to detect transcriptional changes during preoperative RT of rectal cancer and help detect new predictors for response to radiation. A large-scale prospective study is needed to test this hypothesis. To eliminate the risk of increasing toxicity from repeated biopsy, we first conducted a pilot study to assess the safety of pre- and post-RT rectal tumor biopsies, and also the feasibility of detecting gene expression changes on biopsies from irradiated tumors.

MATERIALS AND METHODS

Patients

Patients presenting with locally advanced rectal cancer (T> T2, N0/Nx, M0) and eligible for preoperative RT were enrolled into the study. Exclusion criteria were: anti-coagulant therapy, cardiac valvular disease, and pelvic pain from prior biopsies. All patients gave their informed written consent to the study, which was approved by the University of Nantes Institutional Review Board for human studies.

Biopsies

Patients were delivered 45 Gy (1.8 Gy/fraction, over 5 wk, 5 d per week). Six tumor and 3 normal tissue biopsies were taken from each patient before RT and one hour after a dose of 7.2 Gy (4th fraction) during RT. Patients were assessed for biopsy toxicity (infection or bleeding) during RT. Tumor purity was measured on tumor cell smears.

RNA isolation and microarray procedures

Tissues were frozen immediately in liquid nitrogen and disrupted using a mortar and pestle. Samples were homogenized in lysis buffer using a syringe and needle. Total RNA was prepared using the RNeasy Mini kit (Qiagen, Valencia, CA, United States). The integrity of the RNA was assessed for each sample using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, United States). Double-stranded complementary DNA (cDNA) and labeled complementary RNA were synthesized from the total RNA and hybridized to the Affymetrix Human U133 plus 2 gene chips (Affymetrix, Santa Clara, CA, United States). The chips were further processed and scanned according to the manufacturer’s protocol. The arrays were scanned with a laser scanner and the data was visualized and normalized using the MAS 5.0 Affymetrix software (Affymetrix, Santa Clara, CA, United States). Over- and under-expressed genes were classified by Gene Ontology category[10].

Statistical analysis

Data were normalized according to the MAS5 algorithm. The ratio “gene expression during RT/gene expression before RT” was calculated. Genes with a ratio > 2.5 or < 0.4 in all patients and a false discovery rate (FDR) of < 11%, as estimated by significance analysis of microarrays, were selected.
RESULTS

Seven patients with rectal cancer (median age: 66 years, range 55-84 years) were included in the study but one later refused to participate (Table 1). Six patients underwent biopsy before and during RT. The median time of the biopsy was 1 h after the 4th RT session (0:27-2:12). No grade 2 biopsy-induced toxicity was reported. Surgery was performed at a median time of 6 wk after RT. No grade 2 intra-operative toxicity was reported.

Mean RNA content was 23 µg/biopsy (range 14.37-37 µg/biopsy) before RT and 22.7 µg/biopsy (range 12.35-55 µg/biopsy) during RT (Table 1). Microarray analysis showed that preoperative RT significantly up-regulated 31 genes and down-regulated 6 genes (the full names of the genes are given in Table 2). According to the Gene Ontology project classification, these genes are involved in protein metabolism (ADAMDEC1, AKAP7, CAPN5, PLA2G4A, NEDD4L, NEDD4L2).

Table 1  Patient characteristics

| Patient | Age (yr) | Disease stage | G1 radiotherapy toxicity | Surgical toxicity | Surgical stage | Outcome at 2 yr of follow-up | Tumor purity in biopsies | Mean RNA content (µg) | Pre-RT biopsies | Early-RT biopsies |
|---------|---------|---------------|--------------------------|-------------------|---------------|----------------------------|-------------------------|---------------------|----------------|----------------|
| 1       | 66      | ut2T2Nx       | No                       | No                | ypT2N0        | NED                       | 100%                    | 18.0                | 19.7           |                |
| 2       | 55      | ut3T1N0       | No                       | No                | ypT4N1        | NED                       | 100%                    | 27.0                | 12.6           |                |
| 3       | 84      | ut3T1N0       | No                       | No                | ypT3N1        | MI                        | 100%                    | 21.0                | 35.8           |                |
| 4       | 78      | ut3T1N0       | No                       | No                | ypT2N0        | NED                       | 100%                    | 37.1                | 28.7           |                |
| 5       | 60      | ut3T0N0       | No                       | No                | ypT3N0        | NED                       | 100%                    | 20.0                | 19.4           |                |
| 6       | 67      | ut7T0N0       | No                       | No                | ypT2N0        | NED                       | 100%                    | 14.2                | 19.9           |                |

NED: Non evolutive disease, MI: Metastatic disease; RT: Radiotherapy.

Table 2  Up- or down-regulated genes in rectal cancer biopsies

| Fold | False discovery rate | ID          | Gene title                                           | Symbol |
|------|----------------------|-------------|-----------------------------------------------------|--------|
| Up-regulated genes | 2.649 | 0 | AB22789 | Anterior gradient homolog 3 (Xenopus) | AGR3   |
|       | 3.427 | 0 | AA743462 | - | - |
|       | 2.524 | 0 | NM_006472 | Thioredoxin interacting protein | TXNIP  |
|       | 5.514 | 0 | AB018305 | Spondin 1, extracellular matrix protein | SPON1  |
|       | 2.95  | 7.551 | NM_152315 | Family with sequence similarity 55, A | FAM55A |
|       | 3.289 | 7.551 | AL536553 | Neural precursor cell expressed, developmentally down-regulated 4-like | NEDD4L |
|       | 2.684 | 7.551 | AF056009 | cAMP responsive element binding protein 3-like 1 | CREB3L1 |
|       | 3.111 | 7.551 | AL137063 | A kinase (PRKA) anchor protein 7 | AKAP7  |
|       | 2.717 | 7.551 | AF266504 | Chemokine (C-C motif) ligand 28 | CCL28  |
|       | 2.546 | 8.39 | BF398413 | FERM domain containing 3 | FRMD3  |
|       | 2.637 | 8.39 | AA554045 | Polypeptide N-acetylglactosaminyltransferase 12 | GALNT12 |
|       | 3.047 | 8.39 | NM_030926 | Integral membrane protein 2C | ITM2C  |
|       | 2.775 | 8.39 | AW026379 | Tumor necrosis factor receptor superfamily, member 11a | TNFRSF11A |
|       | 2.609 | 8.39 | RAB27A | RAB27A, member RAS family | RAB27A |
|       | 2.629 | 8.39 | NM_003570 | Cytidine monophosphate-N-acetylneuraminic acid hydroxylase pseudogene | CMATH |
|       | 2.521 | 8.58 | AV728628 | Chromosome 11 open reading frame 32 | C11orf32 |
|       | 2.579 | 8.58 | NM_016992 | Chloride intracellular channel 5 | CLIC5  |
|       | 2.541 | 10.487 | NM_016548 | Golgi membrane protein 1 | GOLM1  |
|       | 2.728 | 10.487 | AW162846 | Calcium/calmodulin-dependent protein kinase II inhibitor 1 | CAMK2N1 |
|       | 2.806 | 10.487 | A639927 | prostate androgen-regulated mucin-like protein 1 | PARM1  |
|       | 2.896 | 10.487 | NM_024709 | Chromosome 1 open reading frame 115 | C1orf115 |
|       | 3.335 | 10.487 | NM_006269 | Solute carrier family 6 member 8 | SLC6A8 |
|       | 2.869 | 10.487 | NM_019062 | Ring finger protein 186 | RNF186 |
|       | 3.174 | 10.487 | AK025181 | Intestine-specific homeobox | ISX    |
|       | 3.968 | 10.487 | AB007899 | Neural precursor cell expressed, developmentally down-regulated 4-like | NEDD4L |
|       | 8.2   | 10.487 | NM_001870 | Carboxypeptidase E | CPE    |
|       | 4.845 | 10.487 | NM_014479 | ADAM-like, decysin 1 | ADAMDEC1 |
|       | 2.56  | 10.487 | BF195709 | Calpain 5 | CAPN5  |
|       | 2.601 | 10.487 | NM_014656 | Programmed cell death 4 | PDCD4  |
|       | 2.931 | 10.787 | AW971415 | cDNA clone IMAGE:5745639 | -      |
|       | 2.787 | 10.787 | NM_003573 | ATPase, Ca** transporting, ubiquitous | ATP2A3 |

Down-regulated genes

| Fold | False discovery rate | ID          | Gene title                                           | Symbol |
|------|----------------------|-------------|-----------------------------------------------------|--------|
| 0.265 | 8.58 | AI578647 | Coagulation factor II (thrombin) receptor-like 2 | F2RL2  |
| 0.356 | 8.58 | BC200951 | Pta1 oncogene homolog, MYC activator | PVT1   |
| 0.322 | 10.787 | AL109839 | Poly(A) binding protein, cytoplasmic 1-like | PABPC1L |
| 0.356 | 10.787 | NM_039778 | S100 calcium binding protein A2 | S100A2 |
| 0.373 | 10.787 | AL041760 | Transcribed locus | -      |
| 0.38  | 10.787 | DR6983  | Peroxidasin homolog (Drosophila) | PXDN   |
CLIC5; CPE; CREB3L1; NEDD4L1; RAB27A), ion transport (AKAP7, ATP2A3; CCL28; CLIC5; F2RL2; NEDD4L1; SLCA6A8), transcription (AKAP7; CREB3L1; ISX; PABPC1; TXNIP), signal transduction (CAPN5; F2RL2; RAB27A; TNFRSF11A), cell adhesion (ADAMDEC1; PXDN; SPON1; S100A2), immune response (CCL28; PXDN; TNFRSF11A) and apoptosis (ITM2C; PDCD4; PVT1)[13]. Up-regulation of 3 genes (CCL28; CLIC5; PDCD4) was detected by 2 different probes and up-regulation of 2 genes (RAB27A; TXNIP) by 3 probes.

**DISCUSSION**

Biopsies taken during preoperative RT for rectal cancer were not associated with enhanced toxicity (infection or bleeding). cDNA micro-array analysis on tumor biopsies uncontaminated by normal tissue was possible provided that the extracted RNA was amplified. Analysis of gene transcription pre- and post-RT detected many up-regulated genes involved in tumor development such as GOLM1 (prostate cancer)[14], CAMK2N1 (a tumor suppressor gene in colon cancer)[15], AGR3 (breast cancer)[16] and PDCD4 (lung and ovarian cancer)[17]. However and contrarily to in vitro studies[18], it did not detect genes thought to be involved in cell repair after radiation-induced damage.

On the other hand, we detected several early response genes mostly involved in stress such as CPE which protects against oxidative stress-induced cell death and ROS-induced cell apoptosis[19], hypoxia-induced TXNIP which is regulated by hypoxia-induction factor[20,21], CREB3L1 which codes for a protein that is cleaved in response to stress on the endoplasmic reticulum[22], and ITM2C which is over-expressed after alpha radiation but whose role is not known[23].

Many genes implicated in ion channel regulation were up-regulated during radiotherapy, such as NEDD4L which regulates sodium channels via the Wnt/beta-catenin signaling pathway during colon carcinogenesis[24], SLCA6A8 which codes for a NaCl-dependent creatine transporter[25], ATP2A3 which encodes an intracellular pump participating in Ca2+ sequestration, and CLIC5, a member of the chloride intracellular channel gene family, structurally homologous to the glutathione-S-transferase superfamily[26].

Among up-regulated genes, we were surprised to find many that are implicated in the immune response, such as ADAMDEC1 (decisin) which plays a key role in the interaction between dendritic cells and germinal center T-helper cells[27], ITM2C which is involved in TNF-induced cell death[28], TNFRSF11A which codes for a protein of the TNF receptor superfamily, CCL28, a member of the small cytokine CC gene subfamily[29], RAB27 which regulates exocytosis of neutrophil granules[30], and PDCD4 (programmed cell death 4) which is regulated by several interleukins (IL-2, IL-15 and IL-12) in natural killer and T cells[31]. This was the only gene of our list that has been reported to be a predictor of response to preoperative radiochemotherapy in pre-treatment biopsies of patients with rectal cancer[32]. It codes for a tumor suppressor protein that inhibits translation initiation factor eIF4A which lies downstream of the AKT/mTOR pathway and plays a role in response to DNA damage[33-35]. PDCD4 mRNA levels during RT may thus be an interesting surrogate marker in studies of mTOR inhibitors plus RT for rectal cancer.

Three of the up-regulated genes we detected (GALNT12, CMAH and SPON1) are involved in metabolic processes and interactions with glycans, and would seem to occupy a key role in triggering an immune response[36]. SPON1 protein belongs the thrombospondin type 1 repeat superfamily of proteins that bind transforming growth factor-[37]. By analogy with SPON2, it might be involved in mechanisms of activation of innate and adaptive immune responses[38].

Apart from PDCD4 and SPON1, we identified at least two other genes that might constitute novel therapeutic targets in preoperative RT for rectal cancer. TXNIP (the gene coding for thioredoxin interacting protein) is a key regulator of redox status. Thioredoxin is released from cells in response to oxidative stress and its plasma or serum level is a good marker of cancer-related oxidative stress. Because thioredoxin mediates redox-induced cell death in colon cancer cells, it is an attractive target for anti-tumor therapy[39]. Thioredoxin inhibitors are currently under investigation[40]. Rab27A gene expression was also up-regulated during RT. Rab27A is a small G protein which regulates secretory activity in colon cancer cells and promotes invasiveness and metastasis in breast cancer cells[41,42]. Novel inhibitors of Rab geranylgeranyl-transferase might thus prove to be inhibitors of rectal tumor cell proliferation and RT enhancers[43]. Interestingly, the highly up-regulated clone 5745639 was found by Blast analysis 2022 bp from the 3’ end of the Ras-related protein Rab-27A.

A limitation of our study is the small number of tumors analyzed. Our results are thus essentially hypothesis generating. Future attention should focus on the genes that have yielded the most robust results (low FDR and detected by several probes).

In conclusion, biopsies taken during early RT sessions may be used for in vivo measurement of tumor sensitivity and have low morbidity. Many genes involved in triggering immune response seem to be expressed during RT. We hypothesize that the changes in gene profiles observed early during RT may help predict rectal tumor response to preoperative RT, whether alone or combined with chemotheraphy. Gene profiling may help: (1) identify predictors of resistance to RT that will enable exclusion of patients likely to be cured by surgery alone; (2) assess the validity of surrogate markers during phase I testing of new radiosensitizing drugs which are used together with RT to treat selected resistant rectal tumors; and (3) define new targets for improving the efficacy of preoperative RT of rectal cancer. To test our hypothesis, larger cohorts of patients are needed and the best time for gene
profiling during and after RT needs to be determined.

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Terminology
DNA microarray is a collection of microscopic DNA spots attached to a solid surface used to measure the expression levels of large numbers of genes in a tumor sample.

Peer review
In this study, the authors examined the transcriptomic changes during preoperative radiotherapy in French patients with rectal cancer. In general, this is a good attempt to seek transcriptomic biomarkers for patients with rectal cancer and can provide important information for clinicians.
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