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Differential Gene-expression analysis of 6 immunogenes in colon cancer

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1 Introduction

Over decades, development of tumor was explained as a succession of genetic modifications leading to an ultimate loss of control and spreading of altered cells or group of cells. However, recent studies renewed interest in cancer microenvironment in general and immune-surveillance in particular. It is known that colorectal cancers (CRC) inducing a potent lymphocytic reaction are associated with improved long-term prognosis. Interestingly, this increase in survival seems independent of patient characteristics or other related molecular variables including KRAS mutation or the number of tumor containing lymph nodes. Several studies showed that analysis of the location, density and functional orientation of immune cells in colorectal cancers was able to define subgroups of patients with similar outcome (1). These studies showed that these immunological data are better predictor of patient survival than the TNM-classification currently used to stage colorectal cancer (2). The activation of the immune system is triggered by a myriad of cytokines, leading to the regulation of numerous genes related to the adaptive immunity, inflammation and immunosuppression. In colorectal tumours the gene expression profiles of 18 immunogenes were analysed (3). These data showed a dominant cluster of co-modulated genes for adaptive immunity and two clusters of genes encoding mediators of inflammation and immunosuppression. An inverse correlation was found between the mRNA level of genes involved in the T helpers 1 (Th1) immunity and recurrence of 75 CRC $p<0.0001$ (1). A strong correlation was achieved by interferon regulatory factor 1 (IRF-1), IFN-γ, CD8a and granzyme B. Patients with a homogenously increased expression of these genes had the best prognosis. IRF-1 is a transcription factor promoting the regulation of IFN-γ, a major cytokine involved in the activation of the adaptive immunity (4). The protein encoded by granzyme B plays a crucial role in the induction of cell apoptosis induced by the T lymphocytes and natural killer cells. CD8 antigen is a glycoprotein on the cell surface found on cytotoxic T lymphocytes whose function is to mediate cell interactions. In fact, CD8 antigen acts as a co-receptor, recognized by the T-cell receptor. CD8a interacts with HLA-A stabilizing interactions between the T-cell receptor on cytotoxic (CD8+) T-lymphocytes and the Class I MHC. IL-15 induces cell activation and proliferation of innate immunity, that is of natural killer cells and regulates memory T-cells by co-acting with IL-2 (5,6). Recent data suggests that a decreased expression of IL-15 is linked to a higher risk of recurrence and a reduced pa-

tient survival, underlining the role of this cytokine in the context of colorectal cancers (7)

Aside from the immune context, the "true" physical environment of tumours could influence its outcome. The pH, the temperature or the partial O2 pressure are stimuli that could influence the regulation of genes implicated in key cellular steps (8). Among such genes, the carbonic anhydrase gene 9 (CA-9) is regulated by hypoxia within the extracellular space. This isoenzyme is a transmembrane and a tumour-associated protein. It seems to be involved in cell proliferation and is one of the most sensitive cellular biomarkers of and a valuable prognostic indicator for overall survival. (9).

Currently, the TNM classification is used to stratify oncological patients into classes of risks based on the histo-pathological features of tumors. However, it offers only a limited predictive value of tumor response to therapy. In addition, the TNM classification cannot be applied pre-operatively on tumor biopsies.

As mentioned above, growing evidences shows that a new score based on the immune contexture around and within the tumor has a prognostic value superior and independent to the TNM classification for CRC. This immunoscore requires the analysis of the surgical specimen. Limited and unpublished data (personal communication Galon and abstracts) are available showing that a surrogate of the immunoscore could be performed on biopsies yet not reaching the statistical significance of the full-range immunoscore.

We assume that the combination of a limited immunoscore on pre-operative tumor biopsies of colon cancer combined with the analysis of the level expression of the six immunogenes previously described will result in a new pre-operative score with good predictive value. This new score will help to identify sub-classes of high-risks patients who will benefit most from neoadjuvant therapies.

This retrospective study analyses the feasibility of quantifying the expression of IRF-1, IFNy, GZMB, CD8, CA-9 and Il-15 in formalin-fixed, paraffin-embedded (FFPE) surgical and biopsy samples.

2 Material and Methods

This study was approved by the cantonal ethic commission.

2.1 Purification of total RNA

The first part of the laboratory work, was to purify the fixed paraffin embedded (FFPE) tissues. A representative FFPE block was selected previously by an experienced pathologist for each patient, including biopsy and surgical specimens. For the biopsies, 10 consecutive sections were cut, including the first and last sections stained with Hematoxilin-Eosin (H&E) according to standard protocol, the morphological evaluation, 4x 5-µm sections mounted on super frost slides for immunohistochemical use and 4x 10-µm sections for molecular analyses (RNA extraction). The same pathologist (Y.P) delineated the borders between tumoral and non-tumoral tissue. Those slides were stored at 4°C in light tight boxes.

All steps of the procedure RNA purification were performed at room temperature and all the centrifugation steps using a centrifuge placed at 15-25 °C. The kit used was the RNeasy® FFPE Qiagen kit and all the procedure were performed according to the manufacturer's protocol. In brief, in the first step the slides were heated at 70C° for 10 mn, then they were placed in two xylol baths for 1 mn each, followed by two EtOH 100% baths for 1mn, then for 15 sec. into each of the four following EtOH solutions at 95% , 70%, 50 % and 30% ; finishing with a 30 sec bath in non-sterile H_2O. Once the slide dried, the tumoral tissue was scraped off from the slide (respecting the previous delimitation histologically determined by pathology of tumoral-non tumoral tissue) and put in a 1.5ml Eppendorf. 150 µl of PDK (proteinase K digestion) Buffer from the kit were added to it and everything was mixed by a quick vortexing. After being centrifuged for 1 mn at 11'000 rpm, 10 µl of proteinase K was mixed gently to the lower phase of the sample that was finally placed on the Eppendorf bloc at 300 rpm and 56C° for an overnight incubation. After 12h the digestion was stopped and a quick centrifugation was made. After another 30mn at 80°C and 300 rpm incubation a spin down (to mix the pellet up and down was made and the sample was transferred into a new tube. After 3 mn on ice, a centrifugation of 15mn in 13500 rpm was made before transferring the super-

natant (without the pellet) into a new tube. In there 16 µl of DNase Booster Buffer and 10 µl of DNase were added and mixed by inverting the tube and briefly centrifuging. After a 15 mn incubation at room temperature, 320 µl of RBC Buffer were added and pipetted up and down. Then, 720 µl of EtOH 100% were added and pipetted up and down followed by the transfer of the sample to a RNeasy® MinElute® spin column, where the sample was centrifuged for 15 sec at 10000 rpm, those last three steps were repeated until the all sample passed trough. After a 500 µl addition of RPE Buffer and a 15 sec centrifugation at 10000 rpm, the flow trough was discarded and let place to another 500 µl addition of RPE Buffer with a 2 mn centrifugation at 10000 rpm, ended with a second discard of the flowthrough. The column was placed in a new collection tube of which the lid was opened for it to be centrifuged at 13500 rpm (full-speed) for 5 mn. After the flowthrough discarded, the column was placed into another new collection tube, where 22 µl of RNase free H_2O was added and centrifuged at full speed for 1 mn.

2.2 RT-PCR

For all samples an RT-PCR was achieved to analyse the expression of 3 housekeeping genes (GAPDH, GUSB and TBP) and 6 genes of interest: CD8a, CA9, IL15, IRF1, GZMB and IFNG.

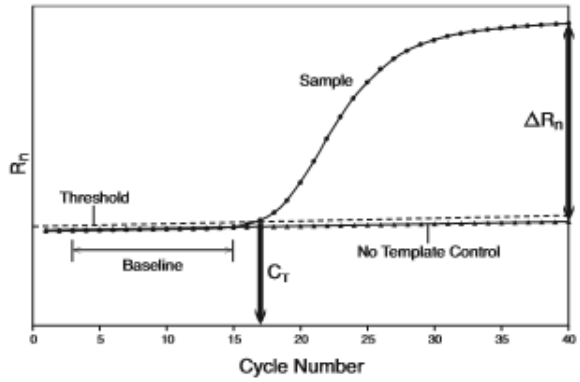
Beginning by the First-Strand cDNA Synthesis Using SuperScript™ II RT.

The whole procedure was performed under strict RNase-free conditions. That is by cleaning the bench using RNaseZap® Solution and by using filter tips and RNase-free H_2O. The SuperScript, RNA and random primers were always kept on ice. First, the RNA and the reagents were thawed and the heat block set at 65°C. 4 µl of 5x First-Strand buffer and 2ul of DTT were mixed per sample to make the Buffer mix. The H_2O, the random primers, the dNTPs and the RNA were mixed, incubated at 65°C for 5 mn and then put on ice. Meanwhile, the heat block was switched to 42°C. The sample was centrifuged briefly and 6 µl of previously made buffer-mix was added, followed by 1 µl of SuperScipt II. This solution was mixed by pipetting up and down. Three incubations were done: 15 mn at room temperature to allow the annealinging of the primers to the single-stranded DNA template, 50 mn at 42°C for the elongation step and 15 mn at 70°C to obtain the final hold. The obtained cDNA could now be used as a template for amplification in a PCR. The cDNA was mixed with the power SYBR green PCR master mix provided by the

manufacturer. RT-PCR was completed by mixing the cDNA first with the primers and then with the polymerase.

2.3 Quantification of cDNA

We used the ABI7900 real time PCR system, where the quantitation of the cDNA relative to a calibrator sample (the calibrator is the sample which all the others will be compared to. It's the untreated sample or the « time zero ». The calibrator's RQ (equals one because it has no variation compare to itself) was measured. The housekeeping genes (GUSB, GAPDH and TBP) being our calibrator sample in this case. A curve was obtained similar to (10):



Different elements were analysed: the baseline: the initial cycles of PCR in which there is little change in fluorescence signal. The threshold: a level of ΔRn—automatically determined (or manually set) by the real-time PCR system software—used for CT determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the amplification plot defines the CT. The threshold cycle (CT): the fractional cycle number at which the fluorescence passes the threshold. The passive reference: a dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or in volume. The reporter dye: the dye used to detect the PCR product. The Power SYBR Green PCR Master Mix uses SYBR® Green I Dye to provide a fluorescent signal that reflects the amount of PCR product. The normalize reporter: the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye. The Delta Rn (ΔRn): the magnitude of the signal generated by the specified set

of PCR conditions (ΔRn = Rn − baseline). Measuring a gene expression, due to the qPCR machine, the amount of specific ARNm in the samples is then obtained. In fact, the machine measures for each cycle the intensity of fluorescence. During the first cycles, there is not enough fluorescence to be detected, but quickly the reaction between the primers and the probe produces more and more amplicons and the exponential curve starts. A PCR curve usually has an exponential period followed by a plateau. The Ct measure has to be taken during the exponential period when the slope is linear. The threshold is placed in that period, and the CT determined where the curve meets the threshold. The curve provides, therefore, the value of the CT that allows us to calculate the rest of the needed values, like: Delta Ct = Ct (gene) – Ct (house-keeping gene), Delta Delta Ct = ΔCt sample 1 – ΔCt calibrator, Delta Ct SD = Standard Deviation (has to be under 0.25. If this value is above 0.25, the RQ value is then considered as being less reliable). RQ = Relative quantification = 2-ΔΔCt. The relative quantification is the Fold change. The calibrator value is fixed at 1. The other samples have a value compare to the calibrator. RQmin and RQmax = possible RQ values defined by the deltaCts SD. (done by the qPCR machine in our study)

2.4 Database

The database served as a basis for information about the patients and was created on Microsoft Excel®. Hundred fifty-five patients were listed on that database where personal information (name, age, sex) and information relative to their colon cancer were inserted as follow: type of tumour, histological samples numbers (surgical and needle biopsy when present), dates of all interventions, hospitalisation times, administration or not of chemotherapy with its date and type, information about the surgical specimen, TNM and outcomes. In order to maintain medical records anonymous, the histological sections were labelled with a code unrelated to patients. For further analyses, data were anonymized.

3 Results

3.1 Patient's cohort

Twenty-four patients operated for colon cancer between 10.09.2011 and 14.08.2013 were selected. 40% were male and 60% were female. In our cohort, the mean age was 71.3 years (std 19.8). The mean follow up was 18.4 months (std 11.7). 20% of the tumours were UICC stage I, 24% stage II, 32% stage III and 24% stage IV (Table 1 (appendix)). During the preoperative period none of the patients received chemotherapy as this could have biased our results. From these 24 patients, 19 samples were surgical specimens and 5 were biopsies

3.2 Genes' expressions

To test the quality of our RNA samples, prototypic housekeeping genes were used. GAPDH, GUSB and TBP were reported to be robust housekeeping genes elsewhere (11). Figure 1 shows the results obtained in our surgical samples. Even if the expression is heterogeneous between samples, the ratio between 2^-Ct values within the same patient is constant, confirming the robustness of these housekeeping genes in our samples. Therefore, these genes were used in the subsequent analysis to normalize our results. Of note, no amplification was obtained for patient 9.

Figure 2 shows the results of RT-PCRs from the six genes of interest: CD8a, CA-9, IFNG, GZMB, IL15 and IRF1.The RNA was extracted from surgical specimen. For each sample, the gene expression was normalized to its mean. Values are shown on a logarithmic scale. The expressions of CD8a, IFNG, GZMB and IRF1 were analysed for every patients. CA9 was not performed in 2 and IL-15 in 5 samples.

The patients' relative mRNA levels are heterogeneous. However, using the Pearson Correlation Coefficient for two sets of values, different correlations between genes were observed. The strongest correlation (0.988) was found between INFG and GZMB (Table 1). A weaker correlation, though still significant, was observed between IL15 and CD8a. Interestingly, a negative correlation was noticed between IFNG and IRF1. According to relative mRNA levels no other genes seem to be correlated

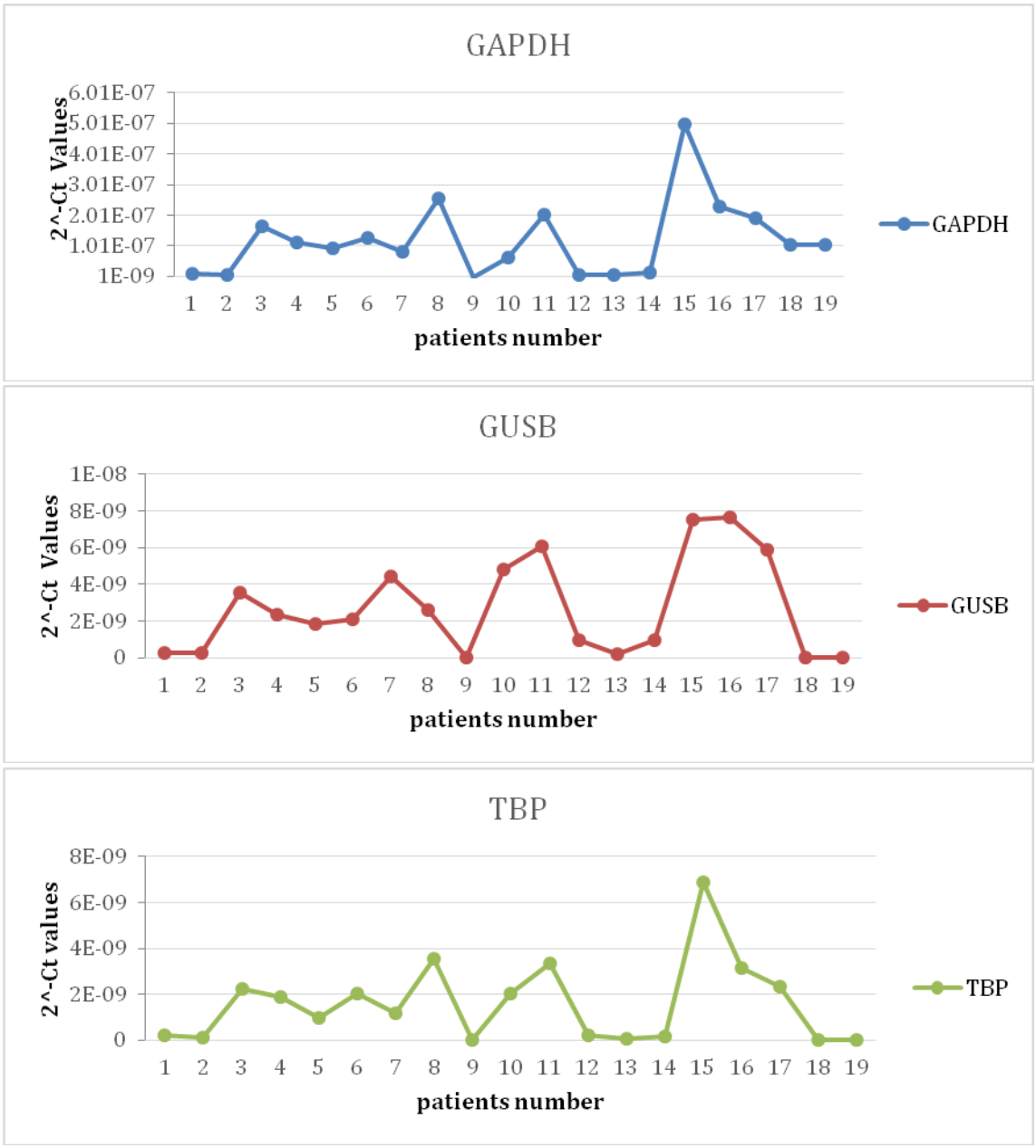


Figure 1. 2^-Ct values of 3 housekeeping genes (GADPH, GUSB and TBP) in surgical samples of different patients. The values of expression show considerable variation between patients, but ratios of 2^-Ct values between the three housekeeping genes are relatively constant. This strongly suggests that gene expression can be reliably measured in stored material of colon cancers.

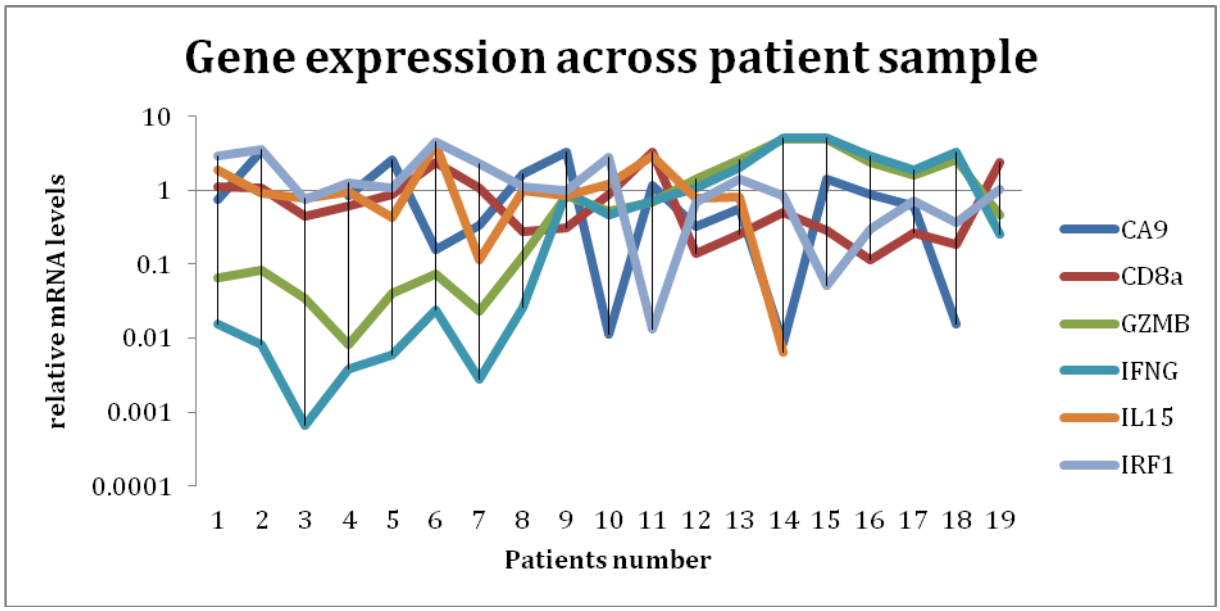


Figure 2. CD8, GZMB, IFNG, IL15 and IRF1 per patient, the expression of each gene was normalized to its mean expression across all patients. Values are shown on a logarithmic scale. In general expression could be measured for all six genes, although CA-9 and IL15 mRNA levels were not detectable in 2/19 and 5/19 patients respectively. Expression of measured genes within one patient, as well as of a given gene between patients appears to be heterogeneous.

	CA-9	CD8a	GZMB	IFNG	IL15	IRF1
CA-9	1.000					
CD8a	-0.087	1.000				
GZMB	-0.190	-0.386	1.000			
IFNG	-0.180	-0.396	0.988	1.000		
IL15	-0.032	0.631	-0.422	-0.431	1.000	
IRF1	0.045	0.304	-0.484	-0.505	0.226	1.000

Table 1. Correlation between the six genes within the same patient cohort. The correlation was determined taking the mRNA levels of expression. A strong correlation occurs between GZMB and IFNG expression (yellow). A weaker, correlation exists between CD8a and IL15. The only negative significant correlation is found between IFNg and IRF1 (red)

3.3 Comparing gene expression and patient outcome

To determine if the expression of our genes was modulated according to tumor stage, two groups of patients were created: those with negative lymph nodes corresponding to UICC stages I and II, and those with positive lymph nodes corresponding to UICC III and

IV. In order to obtain the relative mRNA expression, each 2^-Ct patient's value was used to calculate the mean expression per stage. Figure 4 shows the mean expression of each gene for lymph node negative and positive patients respectively. Interestingly, the relative mRNA expression is higher in patients with positive lymph nodes except for CD8a and CA-9. Both genes show a decreased level of expression while the disease severity increased.

Then, we analysed the expression of mRNA in patients alive with disease and alive without disease. The mean survival time was 18.4 months. The 2^-Ct per gene values was calculated separating them into both categories: alive with disease and alive without disease. As depicted in figure 4, the mRNA levels of expression is higher in patients alive with disease than without disease except for CA-9. The difference of expression between both groups varies according to the genes. Patients alive with disease as compared to patients alive without disease presented higher mRNA expression for IL-15, IRF-1, CD8a, IFNG and GMZB (1.168 , 1.074, 1.415, 1.441 and 1.976 respectively). CA-9 was 2.2 times more expressed in patients without disease than in patients with disease.

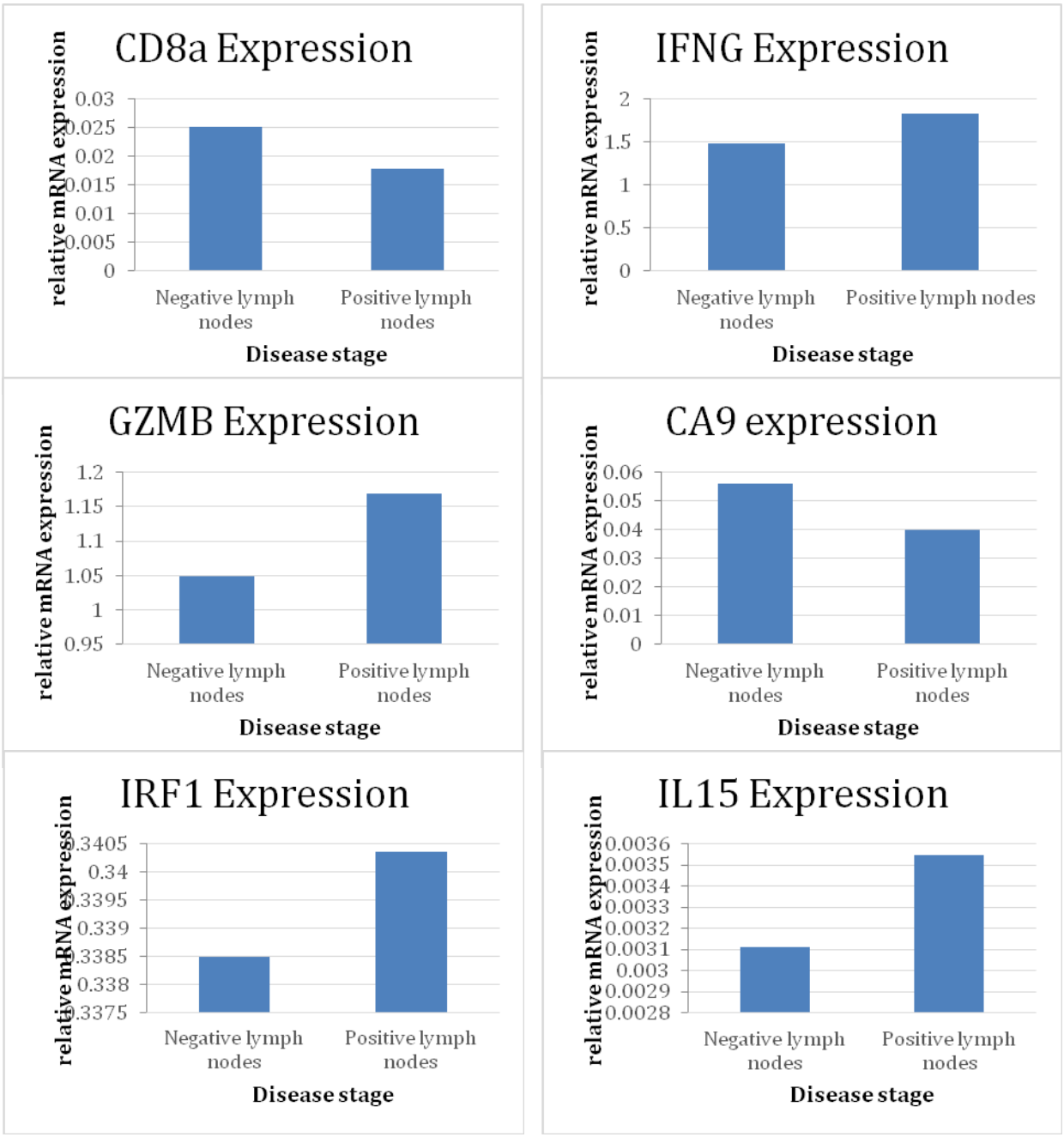


Figure 3. Difference in levels of gene expression according to lymph node stage. For each gene the mean relative mRNA expression of patients is shown, in the first column for patients with negative lymph nodes (stage 1 and 2) and in the second column for patients with positive lymph nodes (stage 3 and 4). IRF1, IL15, IFNG and GZMB are higher expressed in patients with positive lymph nodes. The opposite can be observe for CD8a and CA-9 that have a decrease of their relative mRNA levels with the increase of the disease's severity.

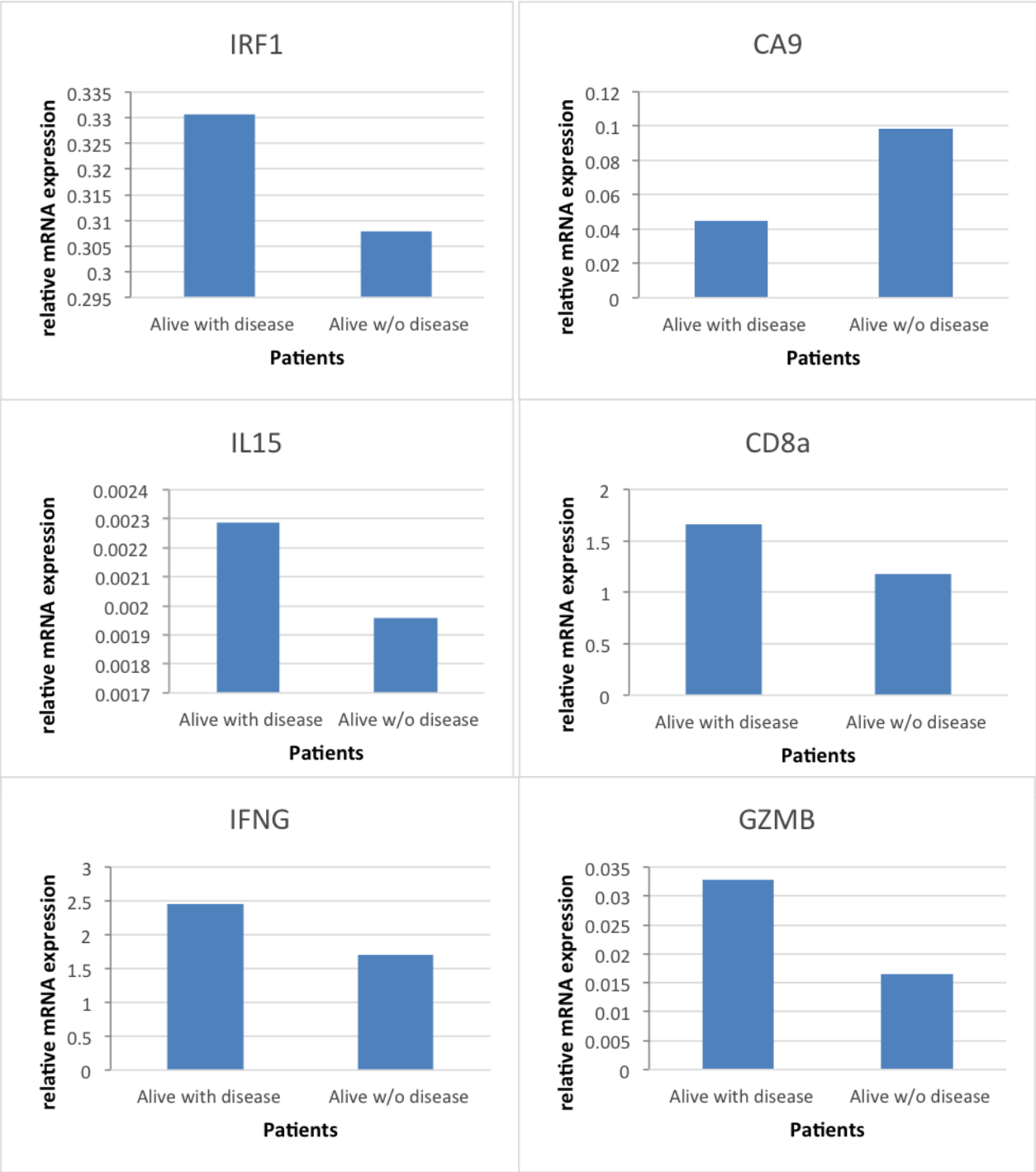


Figure 4. Difference in gene's expression according to disease recurrence. For each gene the mean relative mRNA expression of patients was added, in the first column for patients alive with disease and in the second column for patients alive without disease. the levels of expression decreases for all genes except CA9.

3.4 Comparing gene expression in tumoral and non-tumoral tissue

To determine if our gene of interest showed a different pattern of expression within the tumor and around it, we analysed their expression in both tumoral and non-tumoral tissue. Figure 5 shows the relative mRNA levels for each patient within the tumour and in the surrounding tissue. Again, the 2^-Ct were normalized to the mean 2^-Ct and

shown on a logarithmic scale. The mRNA levels seem to be higher within the tumour than around it, except for CA-9 and IRF1. The levels of heterogeneity between patients for each gene and between genes is very high. To determine if a correlation can be observed between genes in the non-tumoral tissue, we calculated it between genes for all samples. The same procedure was applied for tumoral tissue. Three positive correlation(above 0.75) were observed: between CD8a and IL15, GZMB and INFG and IRF1 and INFG. These results reflect those shown in table 1 and are present in the non-tumoral tissue.

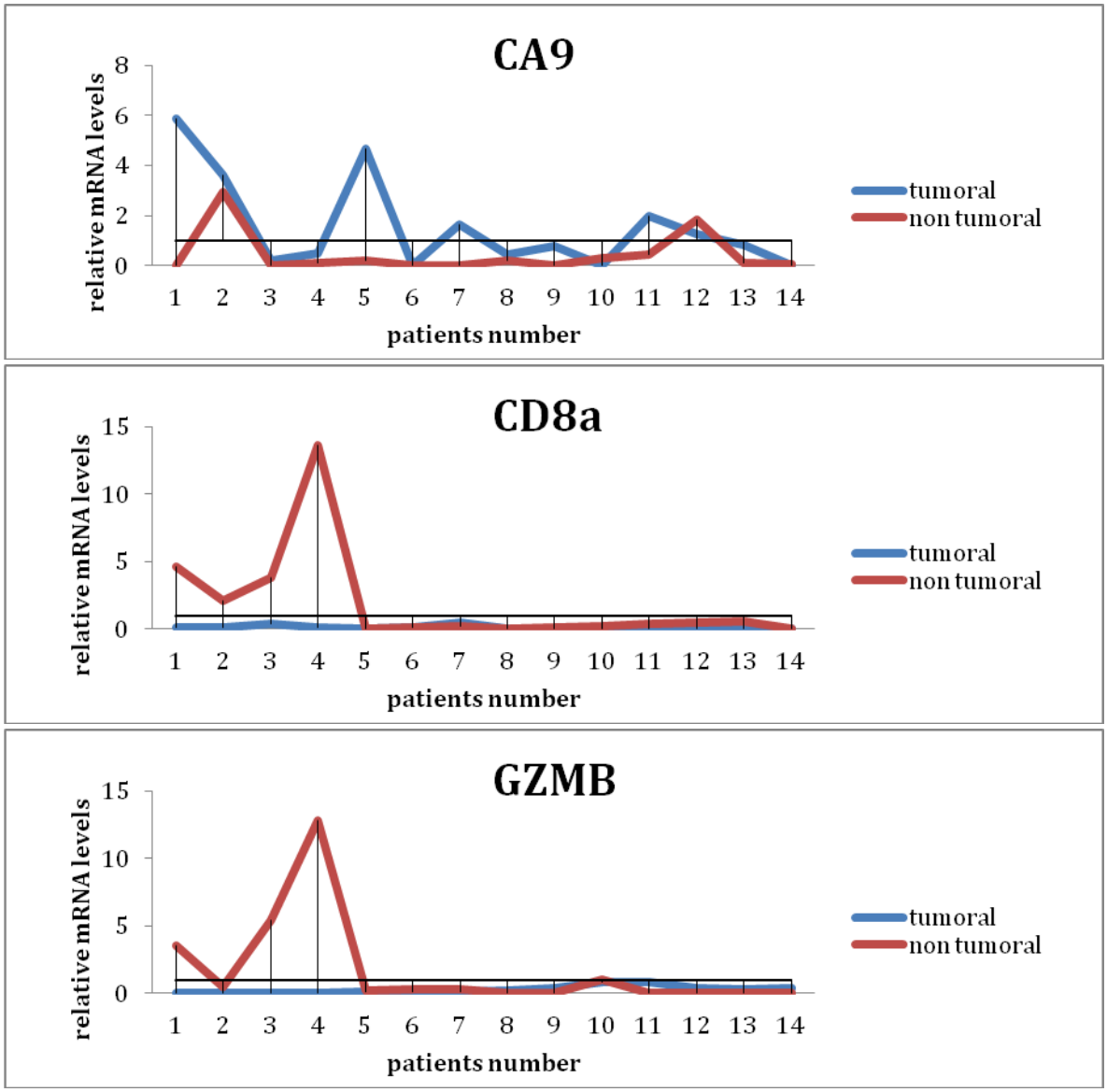


Figure 5 Difference of relative mRNA levels per patient in tumoral tissue and adjacent non-tumoral. For each patient the expression was analysed within the tumor and the microenvironment of the same histological section. The difference between tumoral tissue and microenvironment was done by an experienced pathologist. To obtain their relative value, all expression values were normalized to the mean expression. Except for CA-9 and IRF1 where the mRNA levels tend to be higher in microenvironment than in tumor.

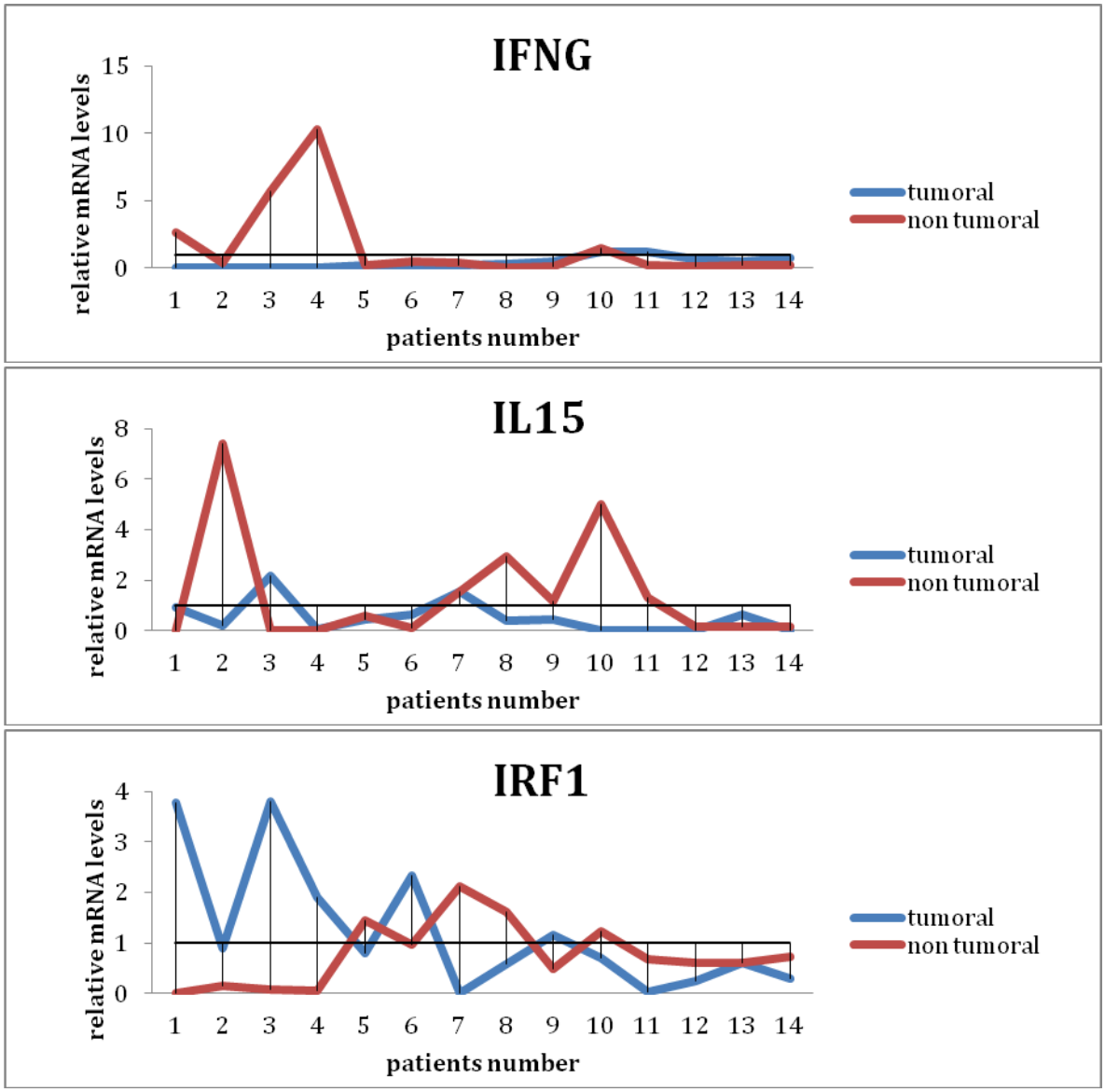


Figure 5 Following

3.5 Gene expression in biopsies

The final step was to determine if gene expression could be analysed in more challenging situation such as tumour biopsies. We studied five biopsies out of the twenty-four patients. The methods used for the RNA extraction and RT-PCR were the same than for surgical samples. As for surgical samples (Fig 1), the first step was to measure housekeeping genes' expression. Figure 6 shows the expression by 2^-Ct values across the five biopsies, for each gene. Compared to figure 1, the gene expression is lower due to the tiny amount of RNA extracted. However, the ratios of 2^-Ct values between the three housekeeping genes are still relatively constant, suggesting that gene expression can still be reliably measured in tumor biopsies. Figure 7 shows the expression of the genes of interest in biopsies: the expression of each gene was normalized to its mean expression

across all patients. Values are shown on a logarithmic scale. Relative mRNA levels, are shown. In general, expression could be measured for all six genes, IL15 mRNA levels were detectable in only two cases. Of note, the undetectable levels occurred in the same gene as in the surgical samples. Also IFNG and GZMB are consistently lower in biopsies than surgical samples. To see if the expression in biopsies were comparable to surgical samples, we calculated the correlation between INFG and GZMB (Fig 8). This analysis shows a correlation similar to surgical samples with a value of 0.65. Figure 8 shows also that gene expression is not only detectable in biopsies but also comparable to trends seen in surgical samples.

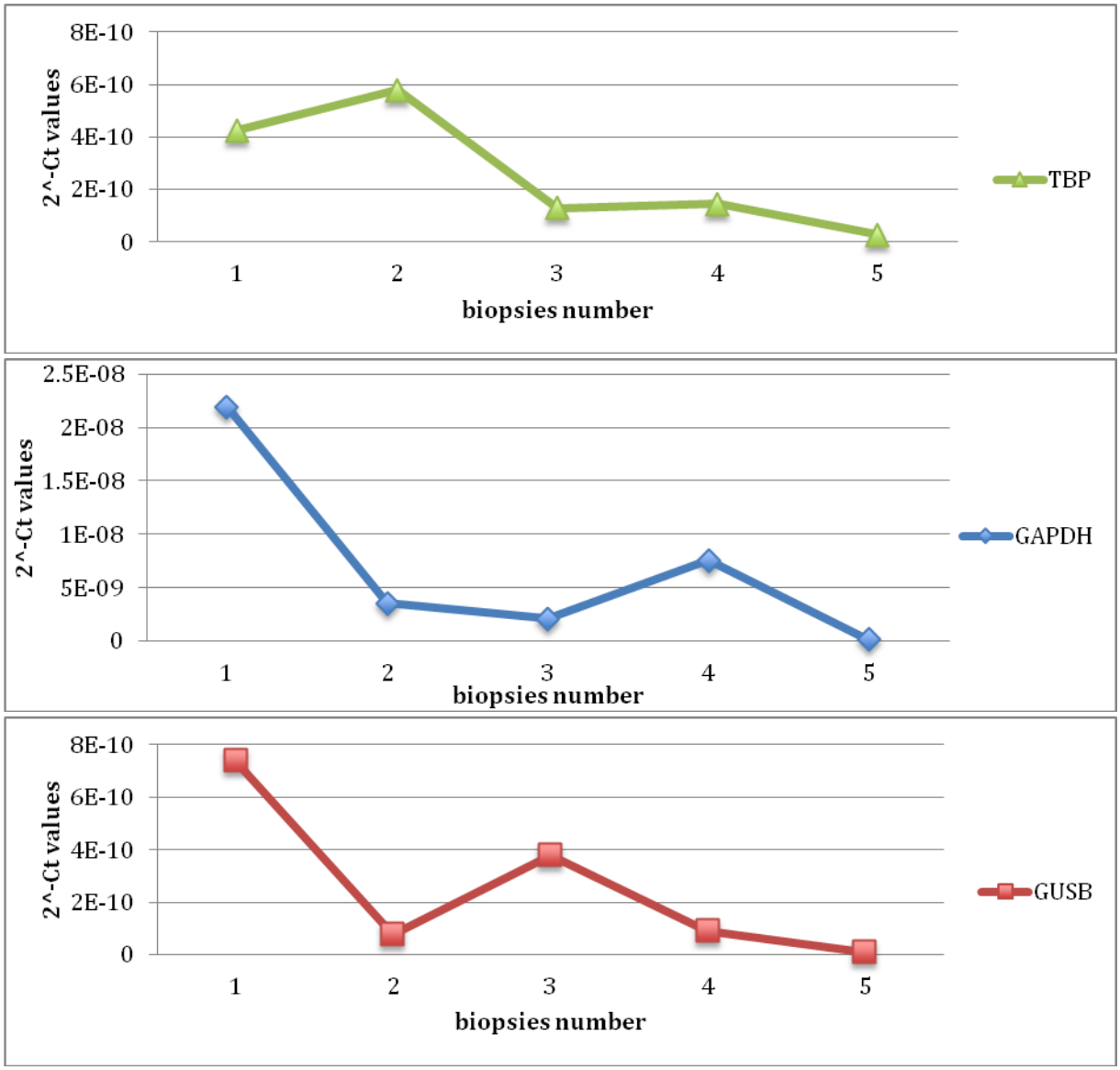


Figure 6. 2^-Ct values for the housekeeping genes : GAPDH, GUSB and TBP for each biopsy. Compared to surgical samples, less mRNA are contained in biopsy specimens. However, ratios of 2^-Ct values between the three housekeeping genes are still relatively constant, suggesting that gene expression can still be reliably measured.

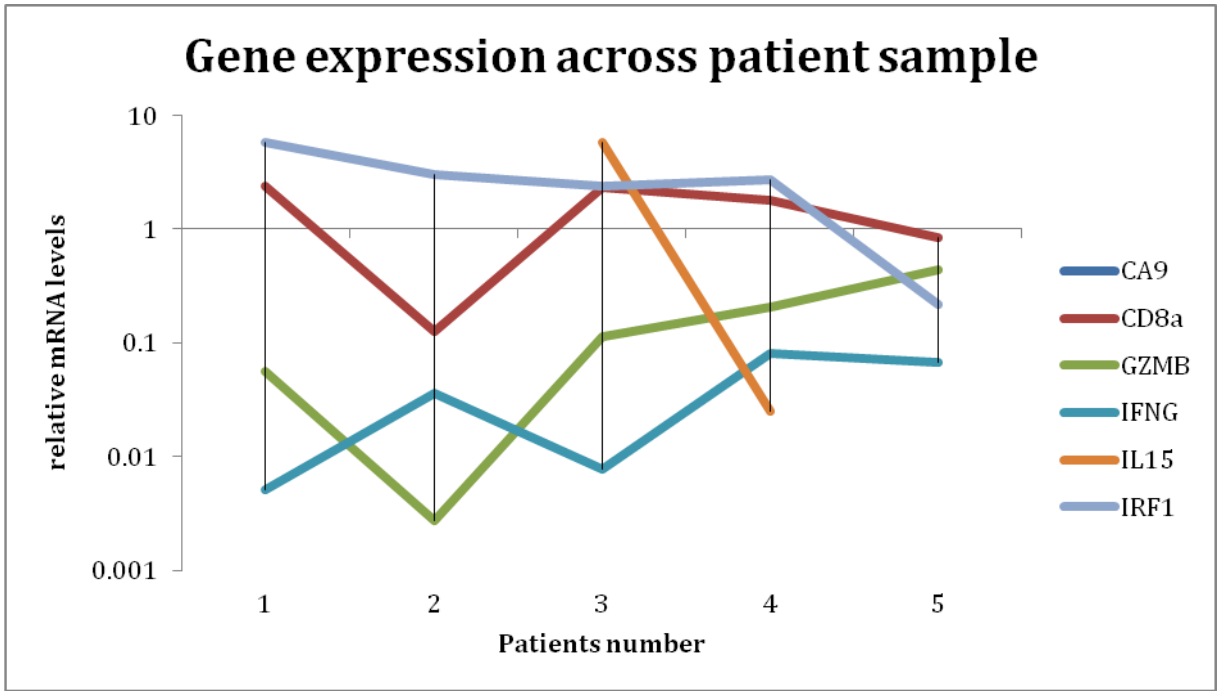


Figure 7. CD8, GZMB, IFNG, IL15 and IRF1 per patient, the expression of each gene was normalized to its mean expression across all patients. Values are shown on a logarithmic scale. Relative mRNA expression of the same genes described above. Relative mRNA levels are shown for five-biopsies samples. In general expression could be measured for all six genes, although IL15 mRNA levels were detectable in only two cases. It is noticeable that the undetectable levels occur in the same gene as in the surgical samples. IFNG and GZMB are consistently lower in biopsies than surgical samples.

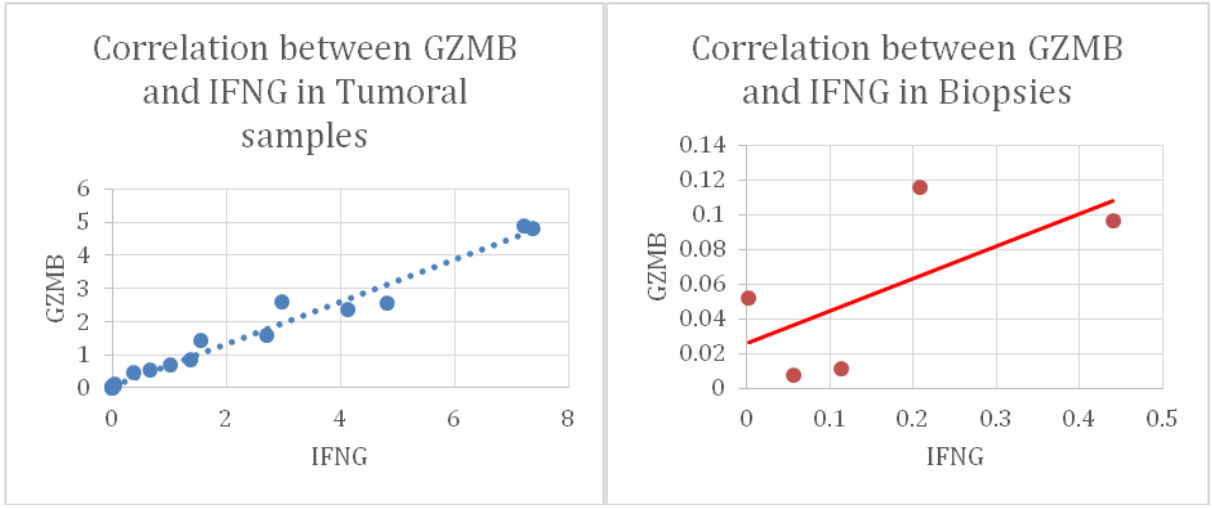


Figure 8. Correlation between GZMB and IFNG in both surgical samples and biopsies. Values of GZMB relative mRNA levels compared to relative mRNA levels of INFG. As shown in figure 3 the positive correlation between GZMB and IFNG is very high in surgical resection samples (left panel). A positive correlation in biopsies (right panel) is also detected, its value being 0.65, showing that gene expression is not only detectable in biopsies but also comparable to trends seen in surgical samples.

4 Discussion

This study analyses the profile of expression of six immunogenes within and around the tumor and in tumor biopsies. No patient received per-operative radio-or chemotherapy that could have biased our results.

A challenging aspect of this work was to set-up protocols able to extract RNA from paraffin-embedded specimen that were stored at -4°C years ago. In fact, RNA extraction is usually performed on fresh frozen material knowing the poor stability of this molecule. Working under strict RNase-free conditions, we were able to extract RNA from FFPE embedded slides at concentration ranging from 10 to more than 500ng/ul depending on the tissue used for extraction (surgical specimen vs biopsies) (data no shown). As we were expected to obtain RNA of average to poor quality, we designed primers with a limited range of amplification on their respective cDNA. GAPDH, GUSB and TBP were reported to be robust housekeeping genes elsewhere (11). Figure 1 shows the housekeeping gene expression on our surgical samples. The values of expression show considerable variation between patients, but ratios of 2^-Ct values between the three housekeeping genes are relatively constant. This suggests that gene expression can be reliably measured in our samples. No amplification was obtained in patient 9, reflecting the complete degradation of its RNA. These 3 genes were then used to normalized the results of mRNA expression of our genes of interest. The expression of each gene was normalized to its mean expression across all patients to identify more precisely the variation of each gene. Thus, we observed a correlation between GZMB and INFG and between CD8a and IL15 (Figure 2). IL-15 and CA-9 amplifications are less robust than the other 4 genes. CA-9 was not amplified in 2/19 patients and IL-15 in 5/19 patients whereas the other 4 genes were amplified.

A recent study showed the impact of IL-15 on colorectal patients. A decreased expression of this gene was embedded with a higher rate of recurrence and metastatic disease (7). One explanation for this decreased expression could be a deletion in chromosome 4, a known feature of advanced colorectal cancers. One of the samples where CA-9 gene expression could not be measured also had an undetectable expression of IL-15 and lit-

tle variation around the mean of expression for the other genes compared to other patients, consistent with poor RNA quality.

The correlation between GZMB and IFNG is high (99%) reflecting their combinatory effects in adaptive and innate immunity at the tumoral site. IFNG is an essential cytokine to mount an immune response against intracellular pathogens and cancer (11). When binding its receptor IFNG activates the JAK-STAT pathway leading to the transcription of numerous genes implicated in the differentiation of naive T cells into cytotoxic T lymphocytes. Those cells along with NK cells will release actively the serine proteases granzyme (13,14).

Another interesting, though weaker correlation exists (Table 1) between CD8a and IL-15. This cytokine is involved in T-cell proliferation. A deletion of the gene containing the IL-15 gene is linked to a significant decreased of T-cells density within colorectal tumours (7). This postulate probably fits our data. In fact, in the 5 patients were IL-15 mRNA was not possible to quantify, 4 had cancer spreading with positive lymph nodes including one that had ovary metastasis.

The intricate interactions between tumor and immune cells reflect the importance of the immune system in controlling tumor spreading. The hypothesis held by Schreiber et al is that most tumor cells are cleared by the immune system (15). If the eradication is not achieved, the immune system will prevent their expansion, creating equilibrium between tumor cell death and proliferation. Rarely, tumor cells will escape the immune vigilance and disseminate beyond the primary site through active mechanisms of immune system repression. This step is crucial for the outcome of patients and the management of patient suffering from tumor outside the primary site is different. The systemic spreading of tumor is often reflected by the presence of tumoral cells within lymph nodes. Thus, the presence or absence of tumoral cells within lymph nodes was used to separate a disseminated disease from a local one. The analysis of the relative mRNA expression of our 6 genes in both groups shows an activation of these immunogenes along with the dissemination of the tumor. Interesting exceptions are CA-9 and CD8a. CA-9 expression is induced by hypoxia (16). To spread outside the primary location tumor cells have to recruit vessels. Cytokines such as VEGF are known promot-

er of vascular development. This vasculature will not only allow the tumor to escape its original location but also will increase the partial O2 concentration reducing thereafter the hypoxia and hence the trigger of CA-9. CD8a level of expression are decreasing raising interesting scenario. T cells need a dual signaling step. The first essential step is the binding of the T cell receptor to its cognate antigen presented by antigen presenting cells (APC)(17). The second signal is the binding of CD28 and CD80/86 on the APC. However, next to CD28, T cells express cytotoxic T-lymphocyte antigen-4 (CTLA-4) that bind the same ligand than CD28 but with higher affinity. It is known that some type of tumor such as melanoma are able to repress the immune system via an action on CTLA-4 as antibody targeting this receptor can lead to spectacular eradication of metastatic melanoma. Hence, one could imagine an active repression of the T lymphocyte by the tumor. Another theory could be the exhaustion of tumor-specific T cells at the site of the primary tumor, as they have to seek tumor cells in remote area. This hypothesis is sustained by the fact that in situ cytokines such as IFNG and IL-15 are more expressed along with the effector granzyme B. This suggests that the T cells within the tumor are more solicited as their number is probably decreasing.

A more global picture of the disease arises when looking the outcome of our patient. Two groups of patients were looked at: those alive with disease and those alive without disease. Interestingly, the pattern of activation of our genes is different showing an increased expression in patients still harboring tumors. CA-9 is again in a reverse situation showing an increased expression in patient without disease. This result is difficult to interpret and needs further evaluation.

A more challenging question was to analyse the variation of mRNA expression within the tumour and around it in the so-called microenvironment. The results show two patterns of expression: the first pattern shows a somewhat similar expression pattern between genes in the microenvironment and in the core of tumour. The relative mRNA levels are more important in the microenvironment than in the tumour. This is true for IFNG, IL-15, CD8a and GZMB. The second pattern shows a disconnection between gene expression in the microenvironment and the tumour. The relative expression level of IRF1 in the tumour mirrors the one in the microenvironment. The level of expression tends to be higher in the tumour for IRF1 and CA-9. As the number of T cells within the

tumour was shown to be less important than around it, both genes should be strongly transcribed to reach such relative level of expression. The expression of CA-9 is congruent with its modus operandi and as expected higher level is found within the tumour (16). IRF-1 is an intra-cellular transcription factor that binds interferon. The expression of IRF-1 is interesting as it could reflects the activation of immune cells that are targeted by IFNG secreted within the microenvironment. A strong correlation between expression of IFNG in patient 3, 4 and 10 can be observed. A strong expression of IRF-1 in one location seems to induce a repression of its expression in the other. To our knowledge no study reported this antinomic activation of IRF1. This result will need further investigation at a cellular level using immunohistochemistry techniques to validate it.

The final aim of our analyse was to see the reliability of such experiments on biopsies. Many difficulties are embedded with tissue-biopsies. First, the amount of tissue harvested in very limited, hence, a limited concentration of RNA after purification. Second, the architecture of the sample is much more heterogeneous: some biopsies contain only tumour other only microenvironment and a last category a mix from the two former. Due to these gross differences between tumoral or non-tumoral tissues, the read-out of the gene expression could be biased, as tiny amount of mRNA of average quality could be generated and no amplification obtained. Figure 6 shows the amplification's profile of the same housekeeping genes used for surgical specimen. As expected the 2^-Ct values for biopsies were 2 logs lower than those for surgical specimen (E^{-8} to E^{-10} and E^{-8} to E^{-6} respectively). Nevertheless, a constant ratio of expression between the three housekeeping genes was observed thereof validating their use to normalize the RT-PCR data performed on biopsies. The gene expression of the 6 genes of interest were analysed in tumour biopsies (Fig 7). All genes were found to have levels of expression except for IL15, which is consistent with the results obtained with surgical samples. In surgical samples, a strong correlation between INFG and granzyme B was observed. This led us to determine if any trend between those two genes could be found in our biopsy samples (Fig 8). Even if the correlation is low (0.65), it correlates with the result found within surgical sample (0.98). The presence of such a trend is encouraging knowing that only five samples of biopsy were analysed. Further work is still needed to validate our results. However, recent data suggest the feasibility of RNA extraction from FFPE biopsy samples (18

5 Conclusions

Growing evidence suggest that immune cells play a pivotal role in tumour development. The immunoscore combine the analysis of different sub-classes of immune cell with patient outcome. It was shown that this score was superior and independent of the TNM classification. However, this immunoscore was performed on surgical specimen only. To date no preoperative therapy is proposed to patient suffering from colon cancer. Surgery remains the only curative therapy for such patients. Moreover, some borderline TNM stages are treated with adjuvant chemotherapy based on non-validated risk factors such as lymphatic or vascular invasion.

Our study is part of a multistep project aimed to identify patients with an unfavourable immunoscore on tumour biopsy. We think that coupling immunohistochemistry with immunogene expression on tumour biopsies could give result comparable to the immunoscore on surgical specimen. These results could lead to a better understanding of the colon cancer and identify patients at risk to develop a worse outcome.

Our results show the feasibility of analysing gene expression from FFPE samples. This was not only possible for surgical specimen but also from tumour biopsies. In addition, we have demonstrated differential expression of genes within and around the tumour with interesting expression profile such as IRF1. To our best knowledge, no such analysis was published.

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8 **Appendix**

Table 1.
TNM Staging Classification of Colon and Rectal Cancer

Stage	Characteristics
Tumor	
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through muscularis propria into subserosa or nonperitonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum
Regional nodal metastasis	
NX	Regional lymph nodes cannot be assessed
N0	No nodal metastasis
N1	Metastasis in one to three pericolic or perirectal nodes
N2	Metastasis in four to more pericolic or perirectal nodes
N3	Metastasis in any node along course of a named vascular trunk and/or metastasis to apical node
Distant metastasis	
MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

(19)