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14

Role of Tumor Tissue Analysis in Rectal Cancer Pharmacogenetics

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1. Introduction

Cancer management has experienced an important progress in the last years due to the discovery of new treatments and an improvement in the early detection methods. These improvements have had an important repercussion in patients´ life span, having an impact in both time and quality life (Berardi et al., 2009). At the same time, knowledge of the specific characteristics of each tumor has led us, in recent times, to be aware of the need of study the unique identity of the cancer (Li & Lai, 2009).

For rectal cancer patients, 5-fluorouracil (5-FU)- based chemoradiotherapy before total mesorectal excision (TME) is the gold standard treatment for stage II and III (Sauer et al., 2004; Wheeler et al., 2004), but the overall rate of response is still about 46-74% (Wheeler et al., 2004; Chen et al.,1994) Research has focused in the discovery of more specific treatments for each cancer and, at the same time, has tried to identify the particular features of cancer cells with the purpose of design target drugs for these cells in order to avoid affect normal cells. Recently, several studies aim at adding to this regimen several different currently available chemotherapeutics in colon cancer treatment, such as the 5-FU prodrug, capecitabine (Carlomagno et al., 2009; Ugidos et al., 2009), oxaliplatin (Carlomagno et al., 2009), irinotecan (Ugidos et al., 2009), cetuximab (Bertolini et al., 2009) or bevacizumab (Willett et al., 2009).

But these treatments are not devoid of adverse effects that could put at risk patients lives due to the treatment itself, so, in these state of affairs,there is a need for identify patients that are going to experience important adverse effects or try to recognize the patients in which the drug benefits will be more than the adverse effects produced; with this purpose, pharmacogenomics and more specific pharmacogenetics studies arise, that so far, have a bright and a dark side.

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In the bright side, there are a few markers with consistent results across studies. Regarding oncology field, hematology has been the pharmacogenomic area with the more important improvement, being several drugs developed for the treatment of different leukemias depending on the genetic of the disease. Development of the first target drug Gleevec supposed an important advance for Chronic Myeloid Leukemia treatment (Buchdunger et al., 1996), and detection of mutations that confer drug resistance (von Bubnoff et al., 2002) allowed to switch to a most favorable treatment depending on the patients´ pharmacogenetics (Hiwase et al., 2011).

Concerning to colorectal cancer treatment and even though it is still necessary to establish a definitive pattern across populations and an extensive research is being realized in that field. From these researches, it has been establish that one of the markers more studied and whose pharmacogenetic association has been more consistently replicated, is high risk of developing severe irinotecan toxicity due to a deficiency in the detoxifying enzyme UGT1A1 (Innocenti et al., 2004; Fujiwara et al., 2010).

Another important detoxifying enzyme related to colorectal cancer treatment is dihydropyrimidine dehydrogenase (DPD). DPD deficiency, the main enzyme related to 5-fluorouracil catabolism, is associated to severe toxicity, patients with this protein deficiency experience mucositis, neutropenia and neurological symptoms under treatment (Johnson & Diasio, 2001; Van Kuilenburg, 2004).

1.1 Germline vs tumoral tissue in pharmacogenetics

But, despite the existence of solid studies supporting the relationship between germline polymorphisms and toxicity of treatment, the efforts of pharmacogenetics studies trying to get information of treatment efficiency from germline polymorphisms have not been as rewarded (Contopoulos-Ioannidis et al., 2006).

One of the genes more studied, mainly related to treatment efficiency but also toxicity is TYMS gene. Polymorphisms in this gene have been associated to different gene expression degree and this to a different protein level (Horie et al., 1995; Kawakami et al., 2001; Mandola et al., 2003, 2004). Although numerous studies have indicated association of germline low-expression alleles in this gene to an increase survival in patients undergoing treatment with 5-Fluorouracil (Mandola et al., 2003; Kawakami & Watanabe, 2003), contradictory results and even no association have been reported. (Showalter et al., 2008)

So far, the only pharmacogenetics markers label by the FDA in colorectal cancer treatments for their study, prior to drug administration, are tumoral expression of EGFR measure by immunohistochemical and KRAS mutation in codon 12 or 13 (FDA, 2011).

Germline pharmacogenetics studies of efficiency are based on the premise of non mutability of the markers in the tumor (McWhinney & McLeod, 2009), nevertheless, being cancer a disease resulting from accumulation of mutations which drives its progression, such assumption, does not appear to have any evidence based support neither from an experimental or literature point of view (Biankin & Hudson, 2011).

To date there is 70 drugs with pharmacogenomic biomarkers in drug labels approved by the FDA. Of these, 21 are oncology- hematology drugs. In Table 1 is shown the kind of information acquired from each gene and the tissue required for its study (FDA, 2011).

The table reflects the utility of the analysis performed in blood related to toxicity but it is noticeable to point out that the FDA recommendations state the necessity of analyze the tumor tissue when performing studies of effectiveness. It remarkable to highlight too, that just a few genes (EGFR, KRAS, Estrogen receptor, Her2/neu and C-kit) are used as

pharmacogenetic markers of effectivity in solid tumors, which indicates that just the association of these genes have been consistently replicated across the studies.

Drug Biomarker	FDA recommendation	Related to	Tissue analyzed
Arsenic Trioxide			
PML/RARα translocation	Positive for PML/RAR-alpha.	Effectivity	Blood
Busulfan			
Chr. Ph	Positive for Philadelphia chromosome.	Effectivity	Blood
Capecitabine			
DPD	Contraindicated in patients with known dihydropyrimidine dehydrogenase (DPD) deficiency.	Toxicity	Blood
Cetuximab			
EGFR	Colorectal cancer. Immunohistochemical evidence of EGFR tumor expression	Effectivity	Tumor
KRAS	Use of Erbitux is not recommended for the treatment of colorectal cancer with KRAS mutations in codon 12 or 13	Effectivity	Tumor
Dasatinib			
Ph+	Positive for Philadelphia chromosome.	Effectivity	Blood
Erlotinib			
EGFR	Patients with EGFR immunohistochemistry (IHC) positive tumors.	Effectivity	Tumor
Fulvestrant			
Estrogen receptor	Hormone receptor positive metastatic breast cancer.	Effectivity	Tumor
Gefitinib			
EGFR	Positive for EGFR	Effectivity	Tumor
Imatinib			
C-Kit	Adult patients with ASM without the D816V c-Kit mutation or with c-Kit mutational status unknown. Patients with Kit (CD117) positive unresectable and/or metastatic malignant GIST. Adjuvant treatment of adult patients following resection of Kit (CD117) positive GIST.	Effectivity	Tumor
Ph+	Newly diagnosed patients with Ph+ CML in CP. Patients with Ph+ CML in BC, AP, or in CP after failure of interferon-alpha therapy. Adult patients with relapsed or refractory Ph+ ALL	Effectivity	Blood

PDGFR gene re-arrangements	Adult patients with MDS/MPD disease associated with PDGFR gene re-arrangements	Effectivity	Blood
FIP1L1-PDGFRα fusion	Adult patients with HES and/or CEL who have the FIP1L1-PDGFRα fusion kinase (mutational analysis or FISH demonstration of CHIC2 allele deletion) and for patients with HES and/or CEL who are FIP1L1-PDGFRα fusion kinase negative or unknown	Effectivity	Blood
Irinotecan			
UGT1A1	A reduction in the starting dose by at least one level of CAMPTOSAR should be considered for patients known to be homozygous for the UGT1A1*28 allele	Toxicity	Blood
Lapatinib			
Her2/neu	Hormone receptor positive metastatic breast cancer that overexpresses the HER2 receptor for whom hormonal therapy is indicated.	Effectivity	Tumor
Lenalidomide			
Chr.5q	Chromosome 5q deletion	Effectivity	Blood
Mercaptopurine			
TPMT	Substantial dose reductions are generally required for homozygous-TPMT deficiency patients and for heterozygous patients when clinical evidences of severe toxicity, particularly myelosuppression, TPMT testing should be considered.	Toxicity	Blood
Nilotinib			
Ph+	Patients positive for Philadelphia chromosome	Effectivity	Blood
UGT1A1	Tasigna can increase bilirubin levels. A pharmacogenetic analysis the (TA)7/(TA)7 genotype was associated with a statistically significant increase in the risk of hyperbilirubinemia relative to the (TA)6/(TA)6 and (TA)6/(TA)7 genotypes.	Toxicity	Blood
Panitumumab			
EGFR	Detection of EGFR protein expression is necessary for selection of patients.	Effectivity	Tumor
KRAS	Use of Vectibix is not recommended for the treatment of colorectal cancer with in patients whose tumors had KRAS mutations in codon 12 or 13.	Effectivity	Tumor
Rasburicase			
G6PD	Do not administer Elitek to patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency.	Toxicity	Blood

Tamoxifen			
Estrogen receptor	Available evidence indicates that patients whose tumors are estrogen receptor positive are more likely to benefit from NOLVADEX therapy.	Effectivity	Tumor
Thioguanine			
TPMT	Inherited deficiency of the enzyme thiopurine methyltransferase (TPMT) may be unusually sensitive to the myelosuppressive effects of thioguanine and prone to developing rapid bone marrow suppression following the initiation of treatment.	Toxicity	Blood
Tositumomab			
CD20 antigen	The BEXXR therapeutic regimen is indicated for the treatment of patients with CD20 antigen expressing relapsed or refractory, low grade, follicular, or transformed non-Hodgkin´s lymphoma, including patients with Rituximab-refractory non-Hodgkin´s lymphoma.	Effectivity	Blood
Trastuzumab			
Her2/neu	Detection of HER2 protein overexpression is necessary for selection of patients appropriate for Herceptin therapy.	Effectivity	Tumor
Warfarin			
CYP2C9 VKORC1	Not all factors causing warfarin dose variability are known. The maintenance dose needed to achieve a target PT/INR is influenced genetic factors (CYP2C9 and VKORC1 genotypes) patients. Dose adjustments are required.	Effectivity	Blood

Table 1. Pharmacogenomic Biomarkers in Drug Labels in Oncology-Hematology labeled by FDA

In line with the pharmacogenetic markers are the pharmacogenetic methods used to test them. A review by Beaulieu et al., make an analysis of the evaluation of the pharmacogenomic tests implemented by some organizations. The authors state: A high degree of heterogeneity between evaluations was observed even within studies evaluating the same pharmacogenomic test¨ (Beaulieu et al., 2010).

Interestingly, of the 44 markers analyzed by the review, only the analysis of HER-2 gene amplification and HER-2 protein overexpression related to the breast cancer treatment, Trastuzumab, and EGFR overexpression related to NSCLC treatment, Erlotinib, was assessed or referred by the four organizations mentioned, and there is only 7 and 10 markers that were evaluated by three and two of them, respectively. This reflects the lack of consensus in the genetic markers utilized for the pharmacogenetic approach of the treatments (Beaulieu et al., 2010).

The analysis, realized by the authors, highlights some issues in some of the studies, like, the poor definition of the genetic group classification used for the evaluation of the markers, as well as the management of the possible false results that were not considered in some of

them. In one of the studies, the authors used inappropriate information to infer the genotype, and in another there is not a clear presentation of the drug dose administrated based on the genetic data. The authors pointed out the need of a confirmatory assay for the evaluation of the markers when a standardized screening method do not exist, confirmation, that it is not always performed. Finally, they underline the confusing assignment of the intermediate phenotype that can lead to a wrong classification of the patients into the groups (Beaulieu et al., 2010).

Regardless these polymorphisms seem to be implicated in the treatment outcome, the lack of replication of these studies together with the fact that most replicated studies are done in tumor samples, bring in relevance the importance of the study of the tumoral tissue (Contopoulos-Ioannidis, 2006; FDA, 2011).

If following the stated lines for this approach, it appears evident that the optimal situation would be the analysis of tumor samples at different times in order to provide updated information enabling a better treatment selection, as it is already done in different leukemias (Baccarani et al., 2006).

However, the difficulty of this practical approach in solid cancers point out the importance of defining the somatic footprinting of the tumor.

Since each tumor has its specific genetic pattern that could be modified because of the addition of new variables, we seek to evaluate the impact of cancer treatments in the modulation of these patterns.

With this aim, following our previous study, where pharmacogenetic markers were studied in pre-treatment tumoral samples, we studied post-treatment tumoral samples in the same cohort of patients with the purpose of try to establish the direction of the somatic mutations under the influence of cancer treatment that we expect will help us, in the future, to find out to find out the mutational mechanisms trigger in rectal cancer that have an impact in the pharmacogenetics markers (Balboa et al., 2010).

1.2 Molecular events produced in a rectal cancer

Even though the adenoma-carcinoma sequence drives the colorectal cancer development (Gloor, 1986), specific molecular events differentiate rectal versus colon cancer (Lindblom, 2001). The proximal colon tumor is more prone to microsatellite instability than rectal and distal areas, whereas distal and rectal colon tumors have been associated with chromosomal instability and microsatellite stability (Li & Lai, 2009; Fernebro et al., 2002; Gervaz et al., 2004).

Other genetic alterations, such as over-expression of *TP53* and *COX-2* genes, and the pattern of mutational frequencies or chromosomal alterations can explain the worse prognosis of patients with rectal cancer (Slattery et al., 2009), but it is noteworthy that patients with different tumors but similar genetic and molecular background seem to have similar survival (Kalady et al., 2009).

In the same way, the existence of mutually exclusive mutations in the same tumor type highlights the importance of differentiate subgroups. These observations reveal the importance of identify the tumor specific genetic pattern (Yeang et al., 2008).

1.3 Pharmacogenomics of Neoadjuvant chemoradiation in rectal cancer

5-Fluorouracil (5-FU), is an antimetabolite of the pyrimidine analogue type which inhibits the DNA and RNA synthesis. The main target for 5-FU is Thymidylate synthase (TYMS); 5-FU acts preventing methylation of the deoxyuridine monophosphate (dUMP) to

deoxythymidine monophosphate (dTMP) by forming a stable complex 5-FU-TYMS, causing a thymine deficiency (Zhang, 2008). The methylation reaction requires the availability of methyl donors, in this case the 5,10-methylenetetrahydrofolate (CH_2THF), which concentration is regulated by several enzymes such as Methylenetetrahydrofolate Reductase (MTHFR) (Gaughan et al., 2000). Since 80-85% of drug catabolic degradation occurs in the liver by Dihydropyrimide dehydrogenase (DPYD) (Ho et al., 1986), deficiency of this enzyme leads to toxicity that can cause death (Johnson et al., 1999). Both the level of TYMS expression (Pullarkat et al., 2001) and the degree of activity of MTHFR (Cohen et al., 2003) have been associated with treatment effectiveness and toxicity, although the latter is mainly related to DPYD activity (Johnson & Diasio, 2001).

Radiotherapy uses ionizing radiation to induce cellular damage either directly or indirectly, through interactions with water-derived radicals causing in DNA both, single-strand breaks and double-strand breaks. Cells that are exposed to radiation start a process that ultimately activate cell cycle checkpoints allowing DNA enzyme repair activity; when DNA damage can not be repaired, cells undergoes apoptosis (Pawlik & Keyomarsi, 2004; Hoeijmakers, 2001). In accordance with the damage generated, different repair systems are working in cells (Hoeijmakers, 2001). Single strands breaks are repaired by a rapid global single-strand breaks repair process, being XRCC1 one of the most important proteins that mediate this process by acting as a molecular scaffold stabilizing and promoting different steps of the single-strand breaks repair process (Caldecotto, 2003): XRCC1 acts direct and indirect by interaction with other molecules in the end processing, gap filling and ligation. Double-strand breaks are repaired by non homologous end-joining, homologous recombination and single-strand annealing, being this kind of damaged which generally leads to a lethal event (Valerie & Povirk, 2003). ERCC1 is an endonuclease of the nucleotide excision repair system that acts not only in the single-strand annealing repair but also there are evidences that acts in the homologous repair of the double strand break (Murray & Rosenberg, 1996; Niedernhofer et al., 2004; Ahmad et al., 2008). Deficiency in this enzyme, and others implicated in the NER system, has also been associated with hypersensitivity to radiation (Parshad et al., 1993; Satoh et al., 1993). One of these enzymes, ERCC2, is implicated in the repair of numerous types of damage and although there are few data on the possible connection between this gene and radiotherapy response it has been hypothesized to participate in the repair of ionizing radiation damage (Rzeszowska-Wolny et al., 2005; Angelini et al., 2005).

Although the volume of the literature on pharmacogenetic markers involved in the response to 5-FU is quite large (Strimpakos et al., 2009; Huang & Ratain, 2009), there are still few studies examining the relationship between pharmacogenetic markers and response to chemoradiotherapy (Lamas et al., 2009), with most of them focused on p53, *Ki-67*, *p21*, and *bax/bcl-2* (Smith et al., 2006; Debucquoy et al., 2006; Kuremsky et al., 2009), cytochrome c oxidase II (COX-2) (Debucquoy et al., 2006), EGF receptor (*EGFR*) (Kikuchi et al., 2009) and TYMS (Kikuchi et al., 2009; Stoehlmacher et al., 2008). A summary of the principal genes studied in relation to rectal cancer are shown in Table 2. However, the clinical utility of these biomarkers remains controversial (Kuremsky et al., 2009), with EGFR, p21 and TYMS as the most validated markers of response until now (Kuremsky et al., 2009).

At the present germline-based pharmacogenetics is useful for predicting toxicity, but has serious limitations for the prediction of treatment response. As stated in a previous study, pharmacogenetic markers should be contrasted with the mutational pattern in each particular tumor type. The study of the tumor and, more specifically, the determination of the tumoral mutational spectrum can possible improve response prediction.

	Gene	Cell function	Mutation or polymorphism	Effect	Hypothesis to test *
Drug CRT (chemoradiation)	**P53**	Implicated in genetic stability, cell proliferation, apoptosis, and inhibition of angiogenesis.	Inactivating tumor mutations	Increased genetic instability and survival of cells with damaged DNA	Loss of p-53 dependent apoptosis and a proliferation advantage. Mutant p53 resistant to CRT
	Ki-67	Asses proliferation.			
	p21	Cyclin-dependent kinase inhibitors that inhibit cells from entering the G1 to S phase.	Tumor mutations		Wild-type p21 suppresses apoptosis in the presence of DNA damage caused by CRT
	bax/bcl-2	BAX is a proapoptotic counterpart of Bcl-2 which inhibits cellular apoptosis.	Bax and Bcl2 expression		Protect cells from radiation-induced apoptosis
	cytochrome c oxidase II (COX-2)	Catalyzes the conversion of arachidonic acid to prostaglandins. These factors are important mediators of tumor invasiveness and metastatic potential.	COX-2 over-expression		Protect tumor cells from damage by generating prostaglandins as tumor survival factors

*(Kuremsky et al., 2009; Gaya Spoverato et al., 2011; Davis et al., 2004)

	Gene	Cell function	Mutation or polymorphism	Effect	Hypothesis to test
Drug 5-FU	**TYMS**	DNA synthesis	5´ 28-bp repeat (rs34743033)	More repetitions increase the efficiency of translation (Horie et al., 1995)	TS protein levels inversely associated with tumor clinical response (Kawakami et al., 2001)
				High- and low-expression haplotypes (Johnston et al., 1994; Mandola et al., 2003)	
			SNP G->C (rs2853542)	High: 2R/3G,3C/3G,3G/3G	Increased survival in low-expression groups (Mandola et al., 2003;
				Low: 2R/2R, 2R/3C, 3C/3C	

				Kawakami & Watanabe, 2003)
		TS1494del6 (rs16430)	Decreases the stability of mRNA (Mandola et al., 2004)	Protective role in adjuvant treatment (Dotor et al., 2006)
	DPYD			
	Drug catabolism	DPYD*2 (IVS14+1 G->A) (rs3918290)	Decreased activity by deletion of exon 14. (van Kuilenburg et al., 2002)	
	MTHFR			
	Implicated in the regulation of the concentration of methyl donors	SNP C677T (rs1801133)	The change Val222Ala leads to a thermolabile variant of MTHFR with reduced enzymatic activity (Frosst et al. 1995)	Increased sensitivity to 5-FU (Sohn et al., 2004; Etienne et al., 2004)
		SNP A1298C (rs1801131)	The change Glu429Ala results in decreased MTHFR activity (Weisberg et al., 1998)	

	Gene Cell function	Mutation or polymorphism	Effect	Hypothesis to test
Radiotherapy Cetuximab	**EGF receptor (EGFR)**			
	Cell proliferation, apoptosis, and differentiation	EGFR overexpression CA-SSR1 (rs11568315)	Approximately 80% inhibition in alleles with 21 CA repeats (Gebhardt et al. 1999)	Response to preoperative radiotherapy (Giralt et al., 2002)
Oxaliplatin Radiotherapy	**XRCC1**			
	Protein that acts as a molecular scaffold, stabilizing and promoting different steps of the SSB repair process, directly and indirectly by interacting with other molecules in the end processing, gap filling and ligation.	Arg399Gln (rs25487)	Changes in binding capacity in the protein with the mutated allele to proteins that interact with it (Evans et al., 1997)	Ionizing radiation hypersensitivity (Hu et al., 2001) Resistance to oxaliplatin (Stoehlmacher et al., 2001)
	ERCC1			
	Endonuclease of the nucleotide excision repair system that acts in the single-strand annealing repair, there is also evidence suggesting that ERCC1 acts in the homologous repair of double-strand breaks	Asn118Asn (rs11615)	Predicts 50% decrease in the efficiency of translation of mRNA to protein (Lunn et al., 2000)	Ionizing radiation hypersensitivity (Lamas et al., 2009) Resistance to oxaliplatin

				(Stohelmacher et al., 2004)
ERCC2 (XPD)				
	Implicated in the repair of numerous types of damage. Although there are few data on the possible connection between this gene and radiotherapy response, it has been hypothesized to participate in ionizing radiation repair damage	Lys751Gln (rs13181)	The wild-type allele exhibits suboptimal radiation-induced damage repair (Lunn et al., 2000)	Possible predictor of clinical outcome (Zárate et al., 2006) Resistance to oxaliplatin (Park et al., 2001)

Table 2. Pharmacogenetic biomarkers in rectal cancer treatment.

2. Material and methods

2.1 Patients & clinical data

We studied germline and tumoral samples of 65 stage II/III rectal patients. They were staged by CT scan, colonoscopy and endorectal ultra-sonography. The tumors were assessed by biopsy. Every treatment began in the 3 weeks following diagnosis and staging. The patients received 5-FU 225 mg/m2/day continuous infusion or capecitabine 825 mg/m2 twice daily during weeks 1–5, along the fractionated radiotherapy schedule (1.89 Gy per day, 50.49 Gy over the whole treat-ment). The surgery was carried out 6–8 weeks after completion of chemoradiotherapy using the TME technique. The surgical procedure included abdominoperineal resection, anterior resection and Hartmann's operation.

Tumor regression was assessed using the tumor regression grading (TRG) system of Mandard *et al,* 1994. as follows:

TRG1: absence of residual cancer and extensive fibrosis;
TRG2: rare residual cancer cells scattered through the fibrosis;
TRG3: increased residual cancer cells but fibrosis still predominating;
TRG4: residual cancer outgrowing fibrosis;
TRG5: absence of regressive changes.

Tumors were classified as good responders (TRG1 and TRG2) or poor responders (TRG3, TRG4 and TRG5). All patients gave written informed consent.

Relevant clinical data were obtained from clinical records (gender, age, TRG and treatment). Response to treatment and overall survival were also analyzed. TRG was assessed by the pathologist in the surgical specimen.

2.2 Genotyping

Genomic DNA was extracted from paired peripheral blood samples and rectal cancer tumors. Blood was obtained before any treatment began, and the tumor used for genotyping was a sample from the initial biopsy. Germline DNA was obtained from leukocytes by peripheral blood samples using a magnetic particle-based purification kit (Chemagen, Baesweiler, Germany). Tumoral DNA was extracted from formalin-fixed, paraffin-embedded sections of the tumor samples after xylene treatment. DNA extraction was performed using the QIAamp® DNA Mini Kit Extraction Column (Qiagen®, CA, USA) in accordance with the protocol. The DNA obtained was rapidly frozen at -20°C.

2.3 Pharmacogenetic polymorphisms

We analyzed a panel of pharmacogenetics markers with previous evidence of relation or possible relation with the treatment currently used in rectal cancer. The pharmacogenetic markers analyzed were polymorphisms in *XRCC1*, *ERCC1*, *ERCC2*, *GSTP1*, *MTHFR* and *DPYD* gene, indicated in Table 2.

2.4 SNaPshot assay

Polymorphisms at *XRCC1*, *ERCC1*, *ERCC2*, *GSTP1*, *MTHFR* and *DPYD* were analyzed by the SNaPshot® (SNaPshot Multiplex System, Applied Biosystems, CA, USA) method. Multiplex PCR primers and SNaPshot probes and methods were previously described (Balboa et al., 2010).

3. Results

Genotyping analysis was performed in 65 enrolled patients of rectal cancer. Their characteristics are shown in Table 3. Median age of the patients was 64 years (range 37-85) and all were submitted to total mesorectal excision (TME). Surgery was scheduled 6-8 weeks after completion of radiochemotherapy. Median time from the end of neoadjuvant treatment and surgery range from 5 to 13 weeks using the total mesorectal excision technique. Patients were divided into two groups according to the neoadjuvant-surgery interval: <8 weeks and ≥8 weeks. Forty-six patients in this study had an interval to surgery ≥8 weeks. Of that group, 20 (43.48%) were good responders. Nineteen patients underwent surgery at an interval <8 weeks and 11 (57.9%) of them were good responders.

Gender	Female	15	(23.1%)
	Male	50	(76.9%)
Age			
	Median (years)	64	
	Range	(37-85)	
Clinical Stage			
	II	20	(30.8%)
	III	45	(69.2%)
Tumor localization			
	Rectal	65	(100%)
TRG	1	19	(29.2%)
	2	12	(18.5%)
	3	20	(30.8%)
	4	10	(15.4%)
	5	4	(6.1%)
Neoadjuvant therapy			
	FU/UFT+RDT	46	(70.8%)
	CAPECIT+RDT	19	(29.2%)

Table 3. Characteristics of the 65 patients

As reported previously (Balboa et al., 2010) no significant associations were observed between good responders in patients operated before 8 weeks compared to those operated after 8 weeks, p=0.297, OR=1.798. The surgery procedure included anterior resection in 39 patients, abdominoperineal resection in 23 patients and Hartman procedure in 3 patients. A histopathologically confirmed complete resection (R0 status) of proximal and distal resection margins was achieved in 62 cases. Tumor regression parameters became apparent by T-level downsizing (comparing pretreatment cT with ypT at surgery) in 46 patients (70.8%). T-level was decreased by one level in 21 patients (32.3%), two levels in 6 patients (9.2%), three levels in 15 patients (23.1%) and four levels in 4 patients (6.2%). UICC downstaging (comparing cUICC and ypUICC) was performed in 49 patients (75.4%).
Sixty-five patients were evaluable for pathological response. Pathological staging was as follows: ypT0N0 19 patients (29.2%), ypT1N0 4 patients (6.2%); ypT2bN0 18 patients (27.7%); ypT2N1 1 patient (1.5%) and ypT3 in 23 patients (35.4%) (N0:11, N1:10; N2:2).
Complete pathologic response TRG1 was observed in 19 (29.2%) of patients and TRG2 was observed in other 12 (18.5%) patients, so the good response rate was of 47.7% in this study. Of the remaining 52.3% of patients, 20 patients (30.8%) showed TRG3, 10 patients (15.4%) TRG4 and 4 patients (6.1%) showed TRG5.
From 65 patients initially studied we obtain tumor samples after treatment in 53 cases. Germline DNA from blood, biopsy samples DNA (T0) and surgical samples DNA (T1) from the patients were genotyped for *XRCC1*, *ERCC1*, *ERCC2*, *GSTP1* and *MTHFR* gene polymorphisms. Genotype distribution in blood is in agreement with that predicted by the Hardy-Weinberg equilibrium. Overall frequencies of the studied polymorphisms were found to be similar to those described in previous reports. A summary of results are in Table 4.

		Blood	**T0**	**T1**
XRCC1	A/A	3	5	1
	G/A	33	42	34
	G/G	29	16	17
ERCC1	C/C	7	3	4
	C/T	31	33	24
	T/T	27	29	23
ERCC2	A/A	23	24	17
	A/C	38	39	33
	C/C	4	1	1
GSTP1	A/A	36	30	26
	G/A	23	21	13
	G/G	6	6	5
MTHFR_C677T	C/C	30	26	19
	C/T	27	28	26
	T/T	8	11	7
MTHFR_A1298	A/A	28	23	20
	A/C	27	35	24
	C/C	10	7	9

Table 4. Genotypes in blood, biopsy (T0) and tumor after treatment (T1)

In Balboa et al. (2010) we described the differences between the genotypes when blood and biopsy are analyzed. When blood sample is used a significant association with response to treatment is given with TS gene 5'UTR, but this significance is lost in the analysis of biopsys, arising an association between better response and genotype AA of XRCC1 gene. However, many differences between the genotype determined in blood and tumor samples were found. Loss of heterozygosity but no microsatellite instability was observed in the study. Some patients, harbouring several mutations and high somatic mutational rate allow us to classify them as hipermutable. The C:G to T:A transitions was the most prevalent changes and C:G to G:C transversions more rare, these percentages, that are conditioned by the initials genotypic frequencies of each gene in the patients cohort, it is driven by the specific mutational mechanisms asociated to each gene in each tumor.

By contrast XRCC1 appeared significant due to the increase of allele A, as results of the transition C:G to T:A. The latter could not happen with the ERCC1 presumably because the allele involved is the C, and that allele is the least frequent. This loss will have little effect on the association analysis, even if such association actually exists.

Table 5 and 6 show the results, of the study of these same patients cohort, after treatment.

As we can observe the tumor after treatment genotypes are more similar to the germline (blood), related to the effectiveness of treatment, able to reverse the genotypes. However a more detailed analysis of data reveals interesting aspects. First, although the genotypes of T1 tumor are more similar to blood, this effect is more pronounced for LOH than for gain of alleles. Yet, for almost all the markers after treatmen we reveal a drop of LOH to 0% except ERCC1 (4.5%) and MTHFR C677 (4.3%). In contrast, individuals who were homozygous in blood and heterozygotes in biopsy (16.9% average, with range from 40% for XRCC1 gene to 8,6% for GSTP1) are reduced in the second tumor in 11.6% (reduction in MTHFR is more pronounced (Balboa et al., 2010).

Thus, regardless of the specific tumor marker and taking into account the possible influence of tissue analyzed (more or less rich in tumor cells) we can broadly see in that reduction of genotype differences (which can be attributed to a reduction of tumor tissue related to tumoral treatment), a clear distinction between the two underlying mechanisms: recovery of LOH and gain of alleles. So, regardless of whether the cells are actually affected by the treatment, is clear that this treatment affects more strongly the former mechanism than the latter. If we establish a connection between genomic instability and LOH versus altered sequence repair mechanisms and gain of alleles seems that a selection is occurring against the first mechanism and not so intense in the second, and the survival cells were those maintain this altered mutational mechanism.

Taking a look at individual markers, it provides valuable information about previously proposed pharmacogenetic hypothesis. So for XRCC1 gene, we have 65 blood, 62 biopsy and 52 resection genotypes. From individuals that were homozygous analyzing blood, we can observe the 11 heterozygous genotypes (AG) in the biopsy analysis and 9 AG genotypes in the second tumor sample. The A allele is described as related to a ineffective protein and consequently associated to a more effective treatment. In our patients there are 4 individuals who revert to a normal (GG) genotype, which would be consistent with the hypothesis but an individual who reverts to AA. Since there have been a reduction in the tumor regression would be expected that cells with A allele would be greatly compromised in their ability to survive. If treatment is not completely effective in GG harbouring cells, the sample should be enriched with G alleles, but not with A ones. Furthermore, 3 individuals whose initial tumor was GG appear after treatment with GA.

	XRCC1		ERCC1		ERCC2		GSTP1		MTHFR_		MTHFR_		Total	
	(%)		(%)		(%)		(%)		C677 (%)		A1298 (%)		(%)4	
LOH[1]														
T0	3	9,1%	2	6,5%	3	8,1%	3	13,6%	6	22,2%	0	0,0%	17	30,4%
T1	0	0,0%	1	4,5%	0	0%	0	0,0%	1	4,3%	0	0,0%	2	8,3%
Gain of allele[2]														
T0	12	40,0%	4	11,8%	5	18,5%	3	8,6%	7	18,4%	8	21,1%	39	69,6%
T1	9	32,1%	3	10,3%	3	15%	1	3,1%	4	13,8%	2	6,7%	22	91,7%
Total[3]														
T0	15	23,8%	6	9,2%	8	12,5%	6	10,5%	13	20,0%	8	12,3%	56	100,0%
T1	9	20,9%	4	9,5%	3	7,1%	1	2,7%	5	11,6%	2	4,5%	24	100,0%

Table 5. Germline changes versus tumor changes (T0 and T1): loss of heterozygosity and gain of alleles in *XRCC1*, *ERCC1*, ERCC2, *GSTP1* and *MTHFR* genes.

	XRCC1		ERCC1		ERCC2		GSTP1		MTHFR_		MTHFR_		Total	
	(%)		(%)		(%)		(%)		C677 (%)		A1298 (%)		(%)4	
Substitutions at C:G base pairs														
C:G>T:A[5]														
T0	14	16,1%	6	13,3%			3	9,4%	10	11,5%			33	58,9%
T1	9	11,5%	4	11,1%			0	0,0%	4	6,0%			17	70,8%
C:G >A:T[5]														
T0					6,0	13,3%					3	6,4%	9	16,1%
T1					2	5,6%					1	2,4%	3	12,5%
Substitutions at T:A base pairs														
T:A>C:G[5]														
T0	1	2,6%	0	0%			3	3,7%	3	7,0%			7	12,5%
T1	0	0,0%	0	0%			1	1,5%	1	2,7%			2	8,3%
T:A >G:C[5]														
T0					2	2,4%					5	6,0%	7	12,5%
T1					1	1,5%					1	1,5%	2	8,3%
Total[6]														
T0	15	26,8%	6	10,7%	8	14,3%	6	10,7%	13	23,2%	8	14,3%	56	100,0%
T1	9	37,5%	4	16,7%	3	12,5%	1	4,2%	5	20,8%	2	8,3%	24	100,0%

Table 6. Germline changes versus tumor changes (T0 and T1): single base substitutions in *XRCC1*, *ERCC1*, ERCC2, *GSTP1*, and *MTHFR* genes.

This case illustrates some of the issues involved in the pharmacogenetic studies:

- Confounding factors. The coexistence of markers related to opposite associations could mask the results (Showalter et al., 2008).
- The association to treatment efficacy may be a statistical artifact, in fact not related to the marker but to the mutational mechanism of the tumor. For example, as already described previously (Sjoblom et al., 2006; Balboa et al., 2010), in rectal cancer the C:C to T:A transitions are the most prevalent changes and consequently the new alleles arising will be mainly A (GG to GA, GA to AA genotypes) but not G (no cases of AA to GA genotypes). Thus, for this marker, the mutagenic mechanism related to the specific G to A changes, determine the appearance of this allele in the tumor and possibly the subsequent association. As we have seen this mutagenic mechanism remains in the tumor after cancer treatment.

Yeang et al. (2008) detected significant different mutational patterns between cell lines and tumor samples. The effect of a polymorphism or somatic mutation in a protein is firstly tested in a cell line. So, another confounding source in the pharmacogenetics studies is due to that data supporting their functional effect come from ¨in vitro¨ studies and the effect observed of these mutations or polymorphisms in the cell lines could not be the same ¨in vivo¨. When these markers are tested in patient samples studies a lack of replication has been observed.

Quantification of the mutations along the different stages could helps us to identify the effective mutations, since it is expected an increase in the population of the cells that carry beneficial mutations for the tumor along the cancer development, but these increase in the number of cells that carry somatic mutations in one stage, but that are not kept across the stages could be explained too by a momentary increase of the uncontrolled cell population that are going to die due to the high number of harmful mutations.

4. Discussion

The difficult of analyzing tumor samples rises from the heterogeneity found in cancer cells that are subdue to different conditions depending on its location in the tumor (Michor et al., 2010). Besides, tumor samples are a mixture of these differents tumor cells and normal cells (Biankin & Hudson, 2011). These circumstances explain the difficult of interpret the results of pharmacogenetic markers in tumor samples.

To analyze tumor samples is important to differentiate too, the genetic background of the patient from the genetic of the tumor, and differenciate these from the response of the tumors to the treatment.

Tumor have an inherent progression, even though this is going to be affected by the patient´s genetic background, there are a pattern of genetic alterations, typical of each tumor. So, when a gene, that are tested in pharmacogenetic studies, is implicated in cancer progression, even though it should be expected a similar trend between patients, different results could be obtained, that are related to the different circumstances that the cells analyzing are being subjected. An example of this is p53, a gene implicated in the adenoma-carcinoma sequence. Overexpression of this gene has been linked to rectal cancer but, analysis of the tumor has shown different expression rates measure by IHC (Kuremsky et al., 2009; Gaya Spoverato et al., 2011). Another example of this is the proliferating cell nuclear antigen (Ki-67) used to assess cell proliferation. A cancer actively growing should have high Ki-67 expression but these will be depending on the stage, the status and the localization of the cells being tested.

Another important point to take into account is that even though cancer treatments are design to act mostly over high proliferative tumor cells, this is dependent of the genetic background of cell tumor. So, although it should be expected that a tumor with high cell proliferative rates, should experience higher efficiency and have a better prognostic, different results can be obtained, depending on the genetic background of cell tumor. If we take as example the meaning of the results of Ki-67, it should be expected that cells with a high proliferative rate, have a high Ki-67 expression, and experience a high treatment efficiency with a good prognostic, but studies by IHC show contradictory results or no correlation with the prognosis, indicating that in tumor cells are being produced a set of different changes that lead to achieve a result which are not explained by the analysis of single markers.(Kuremsky et al., 2009; Gaya Spoverato et al., 2011)
So, to interpret the results from pharmacogenetics studies and to extract information from them, it is of main importance understand the circumstances to which tumor are subjected, and identify the driver mutations, that are produced on them, that will lead its developed and its response to the environment (Stratton et al., 2009)

4.1 Ecology of the cancer

All biological system is affected by the interaction of the environment that surrounds it, and it is the response to signals from that environment a major factor that determines the system behavior (Kenny et al., 2006; Crespi & Summers, 2005).
Tumors, as any other biological system, need to survive and proliferate using the resources from their environment. Thus, the environment, where the different cancers are submerged, will shape the pathways that will be chosen by the cancer for its development. And, the response to the different signals received from the different environments over the progression of the cancer will configure the adjustment of the molecular pathways. These adjustments are executed at different levels, being the genetic level the first step of regulation, mainly through somatic mutations and epigenetic. (Stratton et al., 2009)
In this sense, a plethora of mutational events are shared in cancer but the predominance of one over the others is the specific hallmark of each cancer. Identify and determine the meaning of the changes in these molecular pathways in each cancer is key for understand the mechanisms of cancer progression (Slattery et al., 2009).
With this purpose, tumors have to redirect molecular pathways highly organized and controlled by many checkpoints in order to escape from the self-defense mechanisms, apoptosis, and grow in a not favorable environment. To achieve this aim, cells undergo changes at both phenotypic and genotypic levels that allow cancer cells to overgrow normal cells.
Even within the tumor, cells are subjected to different conditions due to a differential oxygen pressure and nutrients input. These conditions determine the adjustments that cells, according to their localization, have to undergo within the tumor. As the tumor grows, cells, in the core of the tumor, experience a decrease in oxygen and nutrients contribution due to a lack of blood supply. These restricted circumstances cause the switch to an anaerobic metabolism which increase the genetic instability in the cells and induce the segregation of angiogenesis proteins (Allen & Louise Jones, 2011).
At the same time, when tumor gets to a critical mass and the conditions for its development have been exhausted, cells in the tumor periphery initiate changes for its migration to localizations where conditions are more favorable. In this transforming process, cells are

subject to a stochastic number of mutations that have a different fitness for the cell. Harmful mutations will inevitably cause cell death and beneficial mutations will be more or less efficient depending on in which cells and moment these mutations happen (Bindra et al., 2005)

But not only the tumor undergoes changes, this interaction is exerted in both ways, the tumor induces a transformation of its environment for its own benefit, inducing changes in the normal cells that support it (genetic alterations in the normal stroma have also been reported) (Kurose et al., 2001; Nosho et al., 2010). Not only cells from the stroma, hypoxia also plays a role in determining the phenotype of infiltrating monocytes, which have an impact on tumor cell behavior, since the inflammatory response have an effect in tumor progression, that can be either pro-tumoral or anti-tumoral (Allen & Louise Jones, 2011).

5. Concluding remarks

As the cancer evolves, different mechanisms drive their progression. The introduction of an additional variable as it is cancer treatments should have an important impact in cancer behavior.

Cancer treatment research, try to identify specific hallmarks of cancer cells that could differentiate them from healthy cells in order to avoid the adverse effects when these treatments are given to the patients. These differential features, can be the formation of new chromosomal entities as it happens in some leukemias or can be a differential regulation of pathways at different levels that are already acting in normal cells.

The importance of study the tumoral samples, before drug administration, raises from the fact that cancer treatments are design to exert their action in cells where these changes had happen.

Introduction of cancer treatment cause a new alteration in the system, tumoral cells have to respond to a new adverse factor, so they again have to module their behavior in order to survive. Once the treatment is given to the patients, two mechanisms of selection should be acting in cancer cells, mechanisms of selection for tumor progression and mechanisms of selection to survive to cancer treatment.

Cancer treatments have a percent of ineffectiveness that can be due to both, drug inefficiency or inaccurate dose so, the number of cells that persist under the treatments and the time that these cells dispose to rearrange the survival and proliferative pathways for its adaptation to the new conditions, will increase the probabilities of emergence of resistance cells to the drug administrated. Since cancer treatments are design to act principally in high proliferative cells, cells that have acquire the mechanisms to proliferate at a higher rate will be the more affected unless this cells posses any mechanisms to avoid it.

The specific mutational pattern in each gene helps to understand their meaning and the impact of these changes in tumor´s behavior (Kim et al., 2008). Different mutational patterns in tumor progression respond to an adjustment of the tumor to the different conditions and stages, depending on the tumor´s needs, in that sense, different mutational patterns should be expected across the stages.

This approach has been used in several studies, were tumors at different stages have been analyzed. In these studies persistence of somatic mutations detected in the primary tumor through the different stages has been observed, but at different frequencies, indicating, as stated Li Ding et al, that the metastasis arises from a minority of cells in the primary tumor (Ding et al., 2010).The analysis of post-treatment tumor samples helps to analyze if the

mutational mechanisms, produced during tumor development, that were analyzed in pre-treatment samples, persist under the cancer treatment, and what changes the cells have undergone to be resistant to treatment.

6. Acknowledgment

This work was supported by grants from Fundación Ramón Areces and from Fundación Barrie de la Maza (Programa DIANA). The authors appreciate access to CHUS Tumor Tissue Bank from Dr. Jerónimo Forteza.

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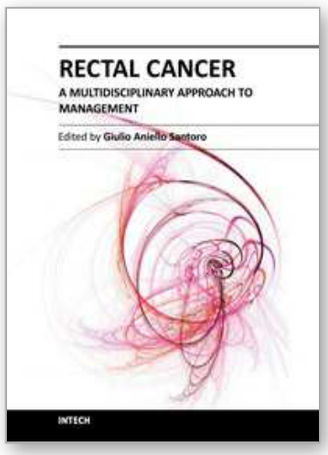
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Rectal Cancer - A Multidisciplinary Approach to Management
Edited by Dr. Giulio A. Santoro

ISBN 978-953-307-758-1
Hard cover, 410 pages
Publisher InTech
Published online 10, October, 2011
Published in print edition October, 2011

Dramatic improvements in medicine over the last few years have resulted in more reliable and accessible diagnostics and treatment of rectal cancer. Given the complex physiopathology of this tumor, the approach should not be limited to a single specialty but should involve a number of specialties (surgery, gastroenterology, radiology, biology, oncology, radiotherapy, nuclear medicine, physiotherapy) in an integrated fashion. The subtitle of this book "A Multidisciplinary Approach to Management" encompasses this concept. We have endeavored, with the help of an international group of contributors, to provide an up-to-date and authoritative account of the management of rectal tumor.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Emilia Balboa, Goretti Duran, Maria Jesus Lamas, Antonio Gomez-Caamaño, Catuxa Celeiro-Muñoz, Rafael Lopez, Angel Carracedo and Francisco Barros (2011). Role of Tumor Tissue Analysis in Rectal Cancer Pharmacogenetics, Rectal Cancer - A Multidisciplinary Approach to Management, Dr. Giulio A. Santoro (Ed.), ISBN: 978-953-307-758-1, InTech, Available from: http://www.intechopen.com/books/rectal-cancer-a-multidisciplinary-approach-to-management/role-of-tumor-tissue-analysis-in-rectal-cancer-pharmacogenetics

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