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CORRELATIVE ANALYSIS OF PUTATIVE MOLECULAR PREDICTIVE FACTORS IN PATIENTS WITH CURATIVELY RESECTED STAGE III COLON CANCER, TREATED WITH ADJUVANT OXALIPLATIN-BASED CHEMOTHERAPY

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Abstract

Colon cancer is the second cause of death for neoplasm worldwide. In most cases it is diagnosed when still localized to the intestinal wall or in regional lymph nodes. Adjuvant therapy with 5-Fluorouracil (5FU) and folinic acid (FA), in combination with oxaliplatin (FOLFOX) are the standard options for patients with radically resected stage III disease. However, a proportion of patients will develop recurrence due to drug resistance and oxaliplatin-based chemotherapy is a regimen that may cause potentially disabling sensory neuropathy. Therefore there is an increase needing for a better selection of patients to be addressed to the most appropriate chemotherapeutic treatment, also in the adjuvant setting.

Several proteins and genetic markers have been described in an attempt to refine prognostic information and predict the benefit derived from systemic treatment. In particular TS protein expression, MSI, p53 expression, *BRAF* and *TP53* mutations, have been described in several reports in relation to 5FU treatment, whereas *ERCC1* polymorphism, ERCC1 expression and *KRAS* mutations, seem to be related to oxaliplatin efficacy in advanced colon cancer patients.

At this purpose we enrolled 230 patients from Argentina and Switzerland who underwent surgical resection, followed by 6-months adjuvant treatment: 106 were treated with 5FU alone and 124 with FOLFOX. In all the cases we investigated the MSI status by fragment analysis, we analyzed *BRAF*, *KRAS*, *TP53* mutations and *ERCC1* codon 118 polymorphism by direct sequencing and we performed ERCC1 expression analysis at protein and mRNA levels by immunohistochemistry and real-time PCR, respectively. Finally, we correlated the molecular and immunohistochemical results with the clinical data.

Above all, a little advantage in survival was observed for patients treated with FOLFOX regimen if compared to those treated with 5FU (51.3 and 41.6 months, respectively, for DFS; 55.4 and 49.3 months, respectively, for OS), although the difference was not statistically significant, probably due to the low number of analyzed cases.

We found MSI in 12% of cases, *BRAF* mutations in 9% of cases, *KRAS* mutations in 28% of cases and ERCC1 resulted over-expressed in 40% of cases detected by IHC and in 49% of cases detected by real-time PCR. These percentages, as well as the types of alterations, are in line with those published in the literature.

Concerning the correlations among markers, we observed a significant association between MSI and *BRAF* mutations (in agreement with the literature) and absence of association between *KRAS* mutations and ERCC1 expression (at odds with the hypothesis proposed in a recent preclinical study).

When we matched the clinical data of the whole patients cohort with molecular alterations, we found a trend towards a better prognosis for patients with MSI than for those with a MSS status (p=0.17); we observed that *KRAS* mutations confer a worse prognosis to advanced colon cancer patients, borderline for the DFS (p=0.07) and statistically significant for the OS (p=0.004); finally we found a trend towards a better DFS (p=0.11) for patients showing low levels of ERCC1 mRNA expression.

When we subdivided the patients on the basis of the received treatment (5FU versus oxaliplatin-based chemotherapy), we observed similar percentages of alterations of all the markers between the two groups. By correlating the molecular alterations with clinical data, we found a trend towards a better survival for MSI patients treated 5FU (p=0.16 and p=0.37 for DFS and OS, respectively), while for FOLFOX patients no clinical differences were found between MSI and MSS cases.

As for *KRAS* mutations, in 5FU group we observed a statistical significant worse DFS (p=0.04) and a trend towards a worse OS (p=0.07) in *KRAS* mutated patients if compared to wild-type patients. In FOLFOX group, no statistical differences were identified between *KRAS* mutated and wild-type cases. Stratifying the population on the basis of *KRAS* mutational status, we noticed that in wild-type patients there was no difference in the clinical outcome in the two treatment modalities. On the contrary, in mutated cases a trend towards a better DFS (p=0.28) and OS (p=0.20) was observed in FOLFOX treated patients if compared to 5FU group.

As regards ERCC1 expression, we found only a trend toward a better DFS (p=0.17) in patients characterized by low ERCC1 mRNA levels when treated with FOLFOX.

As for the last markers, *ERCC1* codon 118 polymorphism (AAT/AAC) and *TP53* mutations, we found percentages of alterations in line with the literature (for ERCC1 polymorphism: TT genotype in 31% of cases, CC genotype in 21% of patients; for TP53: 44% of cases showed at least one mutation). The correlations between these two markers and the clinical outcome are now under evaluation.

In conclusion, looking at the whole cohort, we can confirm a better clinical outcome for adjuvant colon cancer patients treated with FOLFOX regimen with respect to 5FU treatment. MSI could be a useful tool indicating a better prognosis also for advanced colon cancer but its role in predicting 5FU or FOLFOX efficacy remains controversial. In addition, we propose to assess ERCC1 mRNA expression analysis before the administration of oxaliplatin-based chemotherapy, in order to early identify the patients who may benefit the most from this treatment.

Finally, we suggest that *KRAS* mutational status could help clinicians in selecting the best chemotherapeutic treatment in the adjuvant setting: only *KRAS* mutant patients should be treated with a platinum-based chemotherapy, while patients whose tumour is *KRAS* wild-type can be treated with 5FU alone, thus preventing adverse side effect in a consistent number of cases. Our results, of course, deserve confirmations.

Introduction

1.1 EPIDEMIOLOGY AND CANCEROGENESIS

Colon cancer is the second cause of cancer-related death, accounting for over one million new cases per year worldwide (*Parkin DM et al, 2005*). There is no sex predominance in this tumour onset. Approximately 5% of colon cancers occur in patients with a chronic inflammatory disease, such as ulcerative colitis, or belonging to inherited families (*Calvert PM et al, 2002*). Thus, most colon cancers in Western countries are sporadic and are believed to be caused by environmental factors, such as red meat or alcohol consumption, high-fat diet, inadequate intake of fibres, obesity, sedentary lifestyle, diabetes mellitus and smoking (*Cunningham D et al, 2010*).

The incidence of colon cancer is uncommon under the age of 50 years, where it occurs predominantly in patients with a family history. After 50 years, the risk to develop a colon cancer increases exponentially, doubling every decade (*Rim SH et al, 2009*). Indeed two thirds of all colon cancers occurr in patients over the age of 65 (*Everhart JE et al, 2009*).

The disease originates from the epithelial cells lining the colon in the gastrointestinal tract *(Figure 1)*.

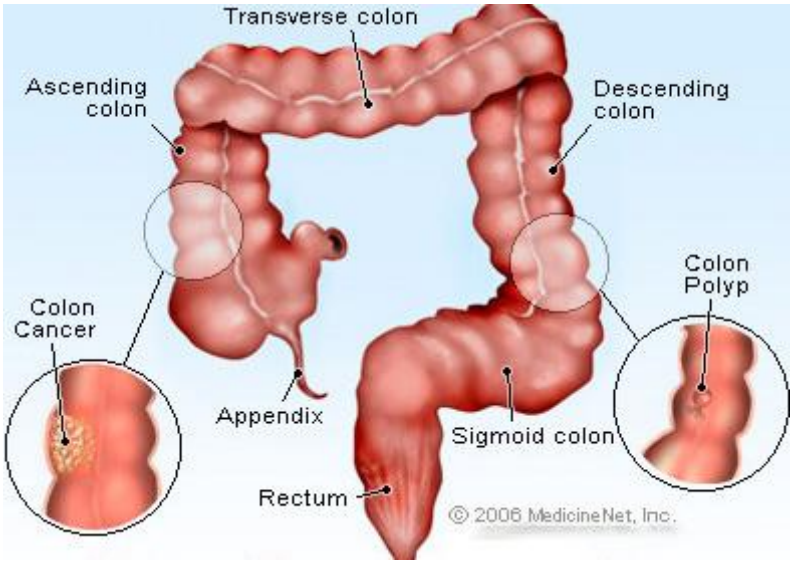

Figure 1: colon cancer (www.medicinenet.com)

Half of all colon-rectal cancers takes place in the colon, with different distribution rates: 16% in caecum and ascending colon, 8% in transverse colon, 6% in descending colon and 20% in the sigmoid colon *(Papadopoulos VN et al, 2004) (Figure 1)*.

Colon cancer does not arise *de novo* but rather is preceded by histological progression from a normal appearing mucosa at risk for colorectal neoplasia to neoplastic tubular and villous adenomas and then to carcinoma formation (*Kronborg O et al, 1999*) (Figure 2). This process takes about a decade or more and is accompanied by a large number of abnormalities in the genes of the colonic epithelium (*Fearon ER and Vogelstein B, 1990*). Two major types of colon cancer, characterized by different carcinogenic processes, have been identified. One, detected for about 15% of colon cancers, is characterized by normal karyotype, normal DNA index and genetic instability at microsatellite loci, and represents the so-called microsatellite instable (MSI) cancer (*Samowitz WS et al, 2005*). The second one, occurring in 80% or more of sporadic colon cancers, was firstly described by Vogelstein, who proposed a tight association between morphology changes and molecular alterations. This second type of colon carcinogenesis suggests that mutations in adenomatous polyposis coli gene (*APC*) represent the initial mutational events that determine hyperplastic proliferation and then early adenoma development. The stage of late adenoma is achieved with *KRAS* mutations, whilst the loss of tumour suppressor genes at chromosome 18q and mutations in *TP53* gene lead to carcinoma in situ and then to the possibility to invade distant organs (the last process is named metastatization) (*Fodde R et al, 2001*) *(Figure 2)*.

Metastatic disease is present in approximately 20% of cases at the time of first colon cancer diagnosis (synchronous metastasis) and in another 30% appears later (metachronous metastasis). The common sites of metastasis are represented by liver (80% of cases), peritoneum and lung *(Venook AP et al, 2004)*.

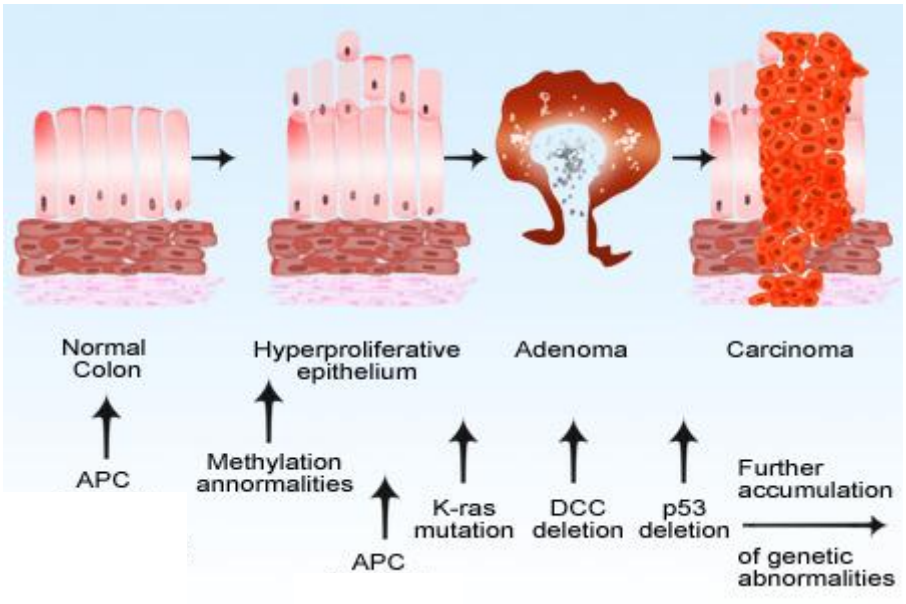

Figure 2: colon cancer formation according to Vogelstein model (www.medicinenet.com).

The two molecular pathways proposed for sporadic cases, are also shared by patients affected by two hereditary syndromes:

1. Hereditary non-polyposis colon cancer (HNPCC), caused by germline mutations in mismatch repair genes, which lead to MSI;

2. Familial adenomatous polyposis (FAP), characterized by germline mutations in *APC* gene (*Benson B, 2007*).

1.2 PROGNOSIS AND TREATMENT

The most commonly used system for staging colon cancer is the TNM, which stands for **T**umor, **N**odes and **M**etastases. This staging system, edited by the American Joint Committee on Cancer, describes the size of a primary tumour (T), whether any lymph nodes contain cancer cells (N), and whether the cancer has spread to another part of the body (M). There are 4 stages of tumour in colon cancer *(Figure 3)*:

- T1: the tumour is only in the inner layer of the bowel;
- T2: the tumour has grown into the muscle layer of the bowel wall;
- T3: the tumour has grown into the outer lining of the bowel wall;
- T4: the tumour has grown through the outer lining of the bowel wall. It may have grown into another part of the bowel, or into other nearby organs or structures, or it may have broken through the membrane covering the outside of the bowel (the peritoneum).

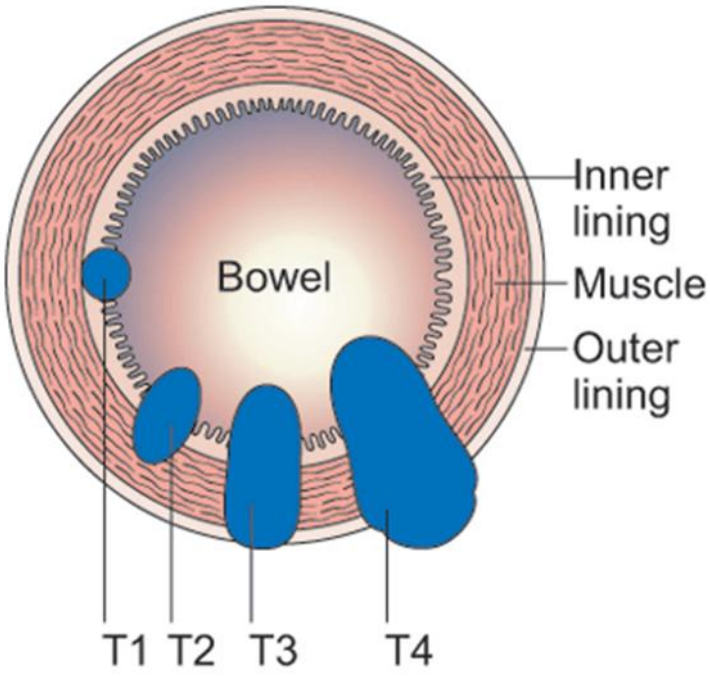

Figure 3: T stages of bowel cancer (www.cancerresearchuk.org).

There are 3 possible stages describing the invasion of tumoral cells in lymph nodes, the presence of neoplastic cells or the number of involved lymph nodes:

- N0: there are no lymph nodes containing cancer cells;
- N1: 1 to 3 lymph nodes have tumoral cells;
- N2: there are cancer cells in 4 or more lymph nodes.

In the case of unknown information concerning lymph nodes involvement, the tumour is classified as Nx.

There are 2 stages of cancer spread (metastasis):

- M0: the cancer has not spread to other organs;
- M1: the tumour has acquired the capability to invade distant organs.

Mx means it is not known if distant organs are involved.

Finally, there are 5 main stages to group colon cancer on the basis of TNM system:

- Stage 1 identifies the cancer localized in the inner layer or in the muscle wall;
- Stage 2 indicates that the cancer has grown up into the outer layer, covering the bowel wall and tissue or organs next to the bowel;
- Stage 3 identifies the involvement of lymph nodes;
- Stage 4 indicates the presence of distant metastases.

Colon cancer is characterized by a significant recurrence rate for which the depth of tumour penetration within the intestinal wall and the presence of involved lymph nodes are major prognostic factors (*AJCC, 2009*). The TNM classification serves as a benchmark for predicting the likelihood of 5-years survival as well as for choosing the best therapeutic option (*Wolpin BM et al, 2007*). In most cases, colon cancer is diagnosed when still confined in the intestinal wall or in regional lymph nodes. Surgery resection is the only curative therapy available for a localised disease; despite of this, approximately 50% of patients die within 5 years from the time of diagnosis and 80% of these will have a detectable recurrence within 2 years. Patients with non-resectable or disseminated disease have a very poor prognosis, with a median survival rate of few months.

Generally, stage I and II tumors are curable by surgical resection alone, and up to 70% of cases exhibiting a stage III disease are curable by surgery combined with adjuvant chemotherapy. Recent advances in chemotherapy have improved survival, but stage IV disease is usually incurable (*Markowitz SD and Bertagnolli MM, 2009*).

Adjuvant chemotherapy for stage III disease mainly includes the use of 5-fluorouracil (5FU) or the more active oral fluoropyrimidine, capecitabine, which has reduced the risk of death by 30%. 5FU can be administered alone or in combination with oxaliplatin (FOLFOX), which improved the 3-years disease-free survival (DFS) (*Midgley R and Kerr DJ, 2005*).

1.3 5-FLUOROURACIL AND OXALIPLATIN

The gold standard chemotherapeutic agents for advanced colon cancer treatment are 5FU and oxaliplatin, two compounds able to increase cell death by interfering with cell functions (*Andrè T et al, 2004*).

5FU is a pyrimidine analogue, belonging to the family of drugs called antimetabolites. It can be administrated also as capecitabine and then converted in 5FU active form after ingestion. It acts in several ways and various mechanisms such as:

- inhibition of thymidylate synthase (TS) by 5-fluoro-20-deoxyuridine-50-monophosphate (FdUMP),
- incorporation of 5-fluorouridine-50-triphosphate (FUTP) into RNA,

- incorporation of 5-fluoro-20-deoxyuridine-50-triphosphate (FdUTP) into DNA,

inducing cell cycle arrest and apoptosis (*Noordhuis P et al, 2004*).

TS inhibition seems to be the main mechanism of 5FU antitumor activity. Interrupting the action of this enzyme, in fact, blocks synthesis of the pyrimidine thymidine, which is a nucleoside required for DNA replication. The administration of 5FU causes a scarcity in thymidine monophosphate (dTMP) levels, produced by deoxyuridine monophosphate (dUMP) methylation due to TS action; so as a consequence rapidly dividing cancerous cells undergo death (*Longley DB et al, 2003*) *(Figure 4)*.

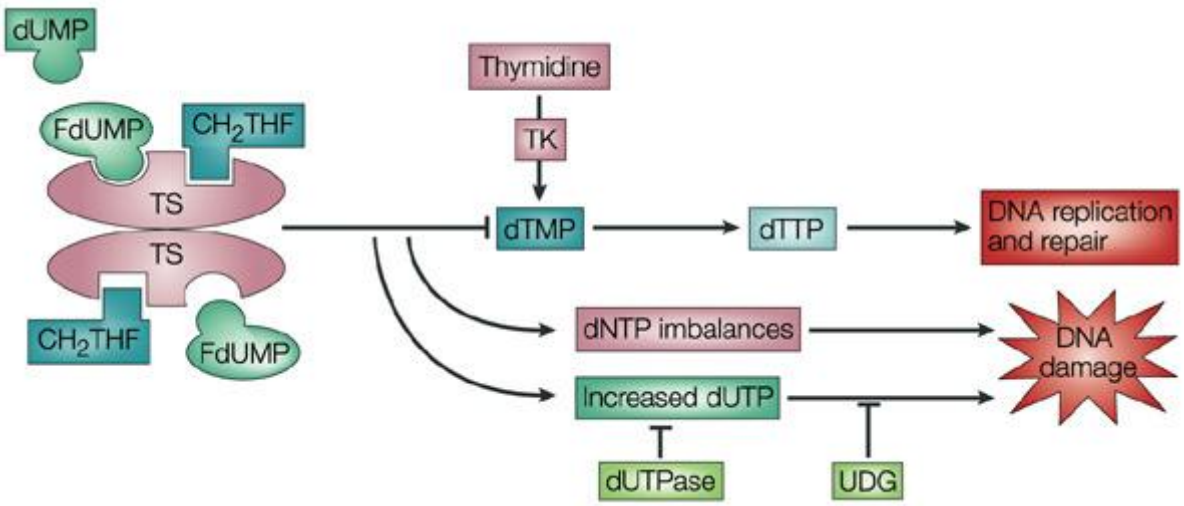

Figure 4: 5-FU mechanism of action (Nature Reviews Cancer, 2003).

Oxaliplatin is a third generation 1,2–diaminocyclohexane platinum analogue with demonstrated preclinical and clinical activity (*Scheeff ED et al, 1999; Vaisman A et al, 1999*). *In vitro* studies have shown that 1,2-diaminocyclohexane-containing platinum drugs belong to a distinct group of cytotoxic compounds with different mechanisms of action and resistance than cisplatin and carboplatin (*Giacchetti S et al, 2000; Sharp SY et al, 2002*). Oxaliplatin interferes with cellular activity by binding to DNA and forming DNA adducts leading to cross-links which disrupt the structure of the DNA molecule, preventing DNA replication and leading to cancer cells death (*Seetharam R et al, 2009*) *(Figure 5)*.

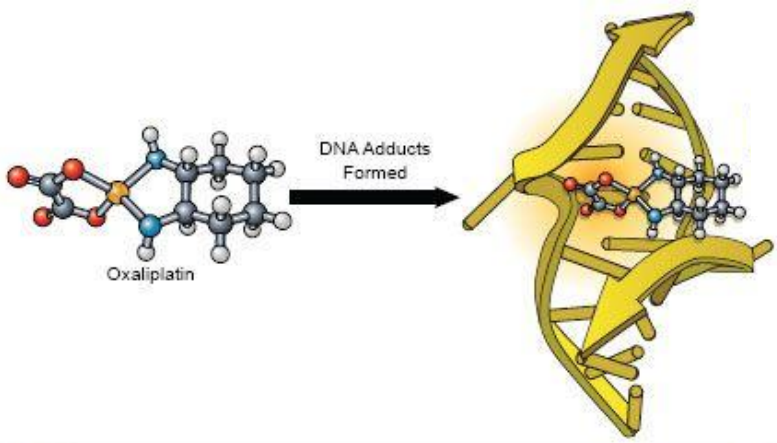

Figure 5: Oxaliplatin mechanism of action (Bhushan S et al, 2009).

1.4 ADJUVANT CHEMOTHERAPY AND ADVERSE EFFECTS

In stage III colon cancer, postoperative adjuvant chemotherapy is the standard of care and it is associated with a statistically significant improvement in disease-free survival (DFS) (p=0.02) and overall survival (OS) (p=0.05) compared to surgery alone, enhancing the 3-year survival up to 71%. The National Surgical Adjuvant Breast and Bowel project (NSABP) trial, reported in 1988, was the first study to show such a survival advantage *(Wolmark N et al, 1988)*.

Before 2000, several new chemotherapeutic agents investigated for antitumor activity in the metastatic colorectal cancer setting were considered promising for the adjuvant treatment. The success in the late 1990s of irinotecan in US trials and of oxaliplatin in European trials in the treatment of mCRC, resulted in the initiation of clinical studies assessing the potential role of these agents in the adjuvant setting. Several phase III trials of oxaliplatin and irinotecan investigated potential improvement of patients outcomes when these agents were administered in combination with 5FU/leucovorin (LV) *(de Gramont A, 2000; Saltz LB et al, 2000; Goldberg RM, 2002; Andre T et al, 2004; Tournigand C et al, 2004) (Figure 6).* In addition, capecitabine was introduced into the adjuvant setting to be investigated for its effect in comparison to bolus 5FU/LV *(Twelves C et al, 2005)*.

Clinical Trial	N	Primary End Points	Disease Stage Included	Trial Conclusions	Reference
INT-0035	929	OS	III	5-FU/levamisole superior to observation	Moertel et al, *J Clin Oncol* 19955
NSABP C-04	2,078	DFS, OS	Dukes B/C	5-FU/LV superior to 5-FU/levamisole	Wolmark et al, *J Clin Oncol* 199910
INT-0089	3,759	DFS	II or III	Equivalency of 6 and 12 month treatment cycles and of high-dose vs. low-dose LV	Haller et al, *J Clin Oncol* 200511
QUASAR	3,238	OS	II	5-FU/LV superior to observation (improves stage II survival by 3.1%)	Gray et al, ASCO 200428
GERCOR C96	905	DFS	Dukes B2/C	Equivalency of LV5FU2 and monthly 5-FU/LV	Andre et al, ASCO 200537
X-ACT	1,987	DFS	III	Capecitabine equivalency with LV5FU bolus; less toxic	Twelves et al, *N Engl J Med* 200519
NSABP C-06	1,553	DFS	II or III	Equivalency of UFT/LV and 5-FU/LV (UFT not approved in US)	Wolmark et al, ASCO 200438
MOSAIC	2,246	DFS	II or III	Superiority of FOLFOX4 to LV5FU2 (improves DFS 3% for all stage II, 5% for high risk stage II)	Andre et al, *N Engl J Med* 200417
NSABP C-07	1,407	DFS	II or III	Bolus 5-FU/LV+oxaliplatin (FLOX) superior to 5-FU/LV	Kuebler et al, *J Clin Oncol* 200724
CALGB 89803	1,264	OS	III	No bolus IFL in stage III adjuvant CRC	Saltz et al, ASCO 200418
PETACC-3	3,278	DFS	II or III	LV5FU2 + CPT-11 not superior (statistically insignificant)	Van Cutsem et al, ASCO 200526

Figure 6*:* Key clinical trials in the adjuvant colorectal carcinoma setting. 5-FU = 5-fluorouracil; CALGB = Cancer and Leukemia Group B; CPT-11 = irinotecan; CRC = colorectal carcinoma; DFS = disease-free survival; FOLFOX4 = 5-fluorouracil/leucovorin/oxaliplatin; GERCOR = Groupe d'Etude et de Recherche en Cancréologie Onco-Radiothérapic; IFL = irinotecan/fluorouracil/leucovorin; LV = leucovorin; LV5FU2 = leucovorin/5-fluorouracil; MOSAIC = Multicenter International Study of Oxaliplatin/5-FU/Leucovorin in the Adjuvant Treatment of Colon Cancer; NSABP = National Surgical Adjuvant Breast and Bowel Project; OS = overall survival; PETACC = Pan-European Trial in Adjuvant Colon Cancer; QUASAR = Quick and Simple and Reliable; UFT = uracil/tegafur; X-ACT = Xeloda in Adjuvant Colon Cancer Therapy *(Marshall JL et al, 2007)*.

Starting from these trials, drug regimens were improved substantially over the years and a 6-months combination of 5FU, folinic acid, and oxaliplatin (FOLFOX) became the reference treatment in 2004 showing a 0.76 hazard ratio (HR) (95% confidence interval [CI] 0.62-0.92) for relapse, favouring FOLFOX compared to fluorouracil plus folinic acid *(André T et al, 2004)*. In a population of stage II-III colon cancer, FOLFOX is associated with a 3-year and 5-year survival rate of 72.2% and 78.5%, respectively. The current consensus is that adjuvant chemotherapy is indicated for stage III colon cancer treatment *(Chau I et al, 2006)*.

Unfortunately, this adjuvant setting is often associated with adverse effects: acute toxicity of the FOLFOX regimen has been well described as moderate to severe *(André T et al, 2004)*. Common adverse effects linked to 5FU use include diarrhoea and heartburn. On the other hand, peripheral sensory neuropathy, in addition to myelosuppression, remains the most common adverse event associated with oxaliplatin, and represents the primary reason for treatment discontinuation. The

toxicity of oxaliplatin affects the peripheral nervous system in two distinct forms. Firstly, it is characterized by an acute reversible sensory neuropathy without persistent impairment of sensory functions. Secondly, it manifests dose-limiting, cumulative, chronic sensory neuropathy *(De Gramont A et al, 2007)*.

Several proteins and genetic markers have been described in an attempt to refine prognostic information and predict the benefit derived from systemic treatment. In particular TS protein expression, MSI, p53 expression, *BRAF* and *TP53* mutations, have been described in several reports in relation to 5FU treatment, whereas *ERCC1* polymorphism, ERCC1 expression and *KRAS* mutations, seem to be related to oxaliplatin efficacy in advanced colon cancer patients *(Popat S et al, 2004; Brody JR et al, 2009; Gavin PG et al, 2012; Lin YL et al, 2012)*.

In the adjuvant setting, many patients must be treated, with significant attendant toxicity, so that a few might benefit. As there are clearly patients who would not have relapsed even without adjuvant therapy, understanding the reasons for treatment failure and developing an ability to predict those who would benefit the most remain important aims in the management of curatively resected colon cancer.

1.5 MSI

As previously mentioned, MSI reflects the inability of the DNA nucleotide mismatch repair system (MMR) to correct errors that commonly occur during the replication of DNA. It is characterized by the accumulation of single nucleotide mutations and length alterations in repetitive microsatellite nucleotide sequences throughout the genome *(Boland CR et al, 1998)*. Several studies have identified MSI as a prognostic factor of better survival: colon cancer patients with MSI showed in fact a significantly better survival if compared to MSS patients (median DFS: 76 and 54 months respectively; $p<0.001$) *(Gryfe R et al, 2000),* data confirmed also in patients with stage II and III colon cancer *(Wright CM et al, 2000; Popat S el al, 2005)*.

On the contrary, there are conflicting data about the role of MSI on the outcome of patients treated with adjuvant 5FU-based chemotherapy. It has been reported that in stage III colon cancer, patients who benefited from 5FU based postoperative treatment showed a MSS pattern, with an increase of 5 months in DFS with respect to MSI patients *(Ribic CM et al, 2003)*. However, in a retrospective analysis of 542 patients

enrolled in different clinical trials and randomly assigned to surgery alone or to adjuvant 5FU, no correlations between MSI status and 5FU therapy were found, either in terms of DSF (p=0.68) or OS (p=0.62) *(Kim GP et al, 2007)*.

Clinical and preclinical studies strongly demonstrated that MMR loss was implicated with resistance to cisplatin *(Brown R et al, 1997; Watanabe Y et al, 2001)*. However, MMR deficiency did not affect the resistance to oxaliplatin, which formed DNA adducts, that are not recognised by the MMR machinery *(Raymond E et al, 2002)*. Nevertheless, the predictive impact of MMR status in patients with stage III colon cancer treated with adjuvant oxaliplatin-based chemotherapy has been explored. It has been observed that patients characterized by MSI showed a great benefit from FOLFOX treatment if compared to MSS patients (p=0.027) *(Zaanan A et al, 2011)*. These data were confirmed by another study where the benefit of MSI patients was detected in FOLFOX group and not in the 5FU one (p=0.01) *(Zaanan A et al, 2010)*.

In a recent study, validating the prognostic impact of MSI in stage III colon cancer patients treated with FOLFOX-based chemotherapy, no difference in the outcome was found between MSS and MSI patients and it has been proposed that the prognostic impact of MMR was dependent on tumour site (*Sinicrope AF et al, 2013)*. Finally, another report showed that MMR status had no relevant predictive value for FOLFOX or 5FU regimens *(Li P et al, 2013)*.

There are still conflicting data about the role of MSI in the adjuvant setting and further analyses must be conducted in order to elucidate the role that MMR machinery can play.

1.6 KRAS

KRAS is a member of the rat sarcoma virus (ras) gene family of oncogenes (including *KRAS*, *HRAS* and *NRAS*), located on chromosome 12, encoding for the guanosine bis/tris phosphate (GDP/GTP)-binding protein RAS, that acts as a self-inactivating intracellular signal transducer *(Bos JL, 1989)*. RAS proteins normally cycle between active GTP-bound (RAS-GTP) and inactive GDP-bound (RAS-GDP) conformations *(Figure 7)*.

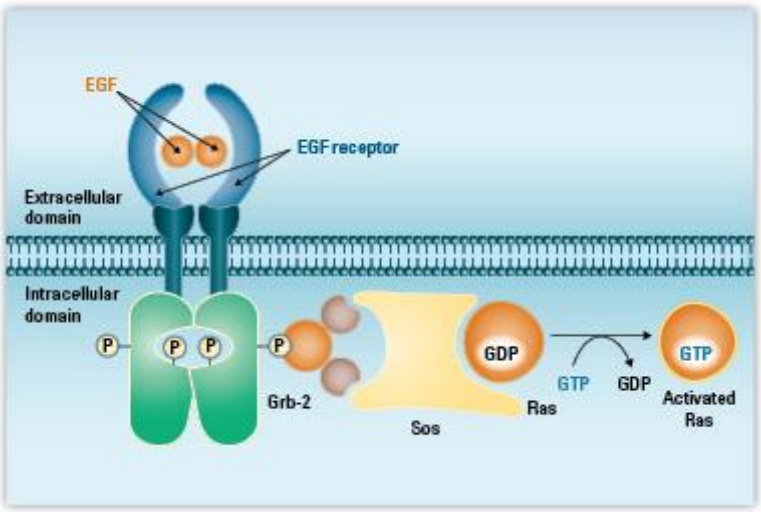

Figure 7: KRAS mechanism of action, active and inactive conformations (www.clarientinc.com).

RAS proteins are activated by guanine nucleotide exchange factors (GEFs) which are recruited to protein complexes at the intracellular domain of activated receptors. Signalling is terminated when RAS-GTP is hydrolized to the RAS-GDP inactive complex by GTPase-activating proteins (GAPs) *(Bos JL, 1989)*.

After binding and activation by GTP, RAS recruits the protein encoded by RAF oncogene, which phosphorylates Mitogen-Activated Protein Kinase Kinase-1 (MAP2K-1), thus initiating the MAPK signaling that ultimately leads to the expression of proteins playing important roles in cell growth, differentiation, and survival. Under physiological conditions, RAS-GTP levels *in vivo* are tightly controlled by the counterbalancing activities of GEFs and GAPs.

KRAS gene mutations are one of the most common alterations in human cancers. In particular, this gene is mutated in over 30% of colon cancers *(Edkins S et al, 2006)*. Mutations within the *KRAS* gene result in RAS proteins that are permanently in the active GTP-bound form due to defective intrinsic GTPase activity leading to a constitutive activation of the downstream signalling. There are limited numbers of mutations in the *KRAS* gene, and altogether more than 90% involve two specific codons (12 and 13). Of these, the most frequent alterations are detected in codon 12 (about 80% of all reported *KRAS* mutations). Codons 12 and 13 somatic missense mutations lead to single amino acid substitutions in KRAS catalytic domain resulting in a constitutive activated form of the protein *(Kosaka T et al, 2004)*.

In metastatic colon-rectal cancer, *KRAS* mutations are established to be predictors of resistance to EGFR-targeted therapies *(Lièvre A et al, 2008)*, but nothing is known about their role in the adjuvant setting.

Preclinical data suggest that response to 5FU may be predicted by *KRAS* mutational status: a transient expression of the mutant *KRAS* gene, but not of the wild-type form, enhanced 5FU-induced apoptosis in colon cancer cells. It seems to occur by the negative regulation of gelsolin, a protein with anti-apoptotic activity *(Klampfer L et al, 2005)*.

Several analyses of recent randomized trials on metastatic colon cancer suggested that the *KRAS* gene mutation status might predict the efficacy of cytotoxic chemotherapy, especially for oxaliplatin-based regimens. OPUS and PRIME studies, which were both designed for patients to receive first-line oxaliplatin/5FU/leucovorin with or without anti-EGFR monoclonal antibodies, are good examples *(Bokemeyer C et al, 2009; Douillard JY et al, 2010)*. These 2 studies showed that DFS in the *KRAS* mutant group lasted longer than that in the wild-type group, with 8.6 versus 7.2 months in the OPUS study, and 8.8 versus 8.0 months in the PRIME study. By contrast, in CRYSTAL study, which was designed for patients receiving first-line irinotecan/5FU/leucovorin with or without EGFR monoclonal antibody, a similar phenomenon was not observed *(Van Cutsem E et al, 2009)*. The median first-line DFS in *KRAS*-mutant and wild-type patients was 7.7 and 8.4 months, respectively.

These studies were the starting point for other preclinical data, focused on the possibility to predict oxaliplatin sensitivity on the basis of *KRAS* mutations. In fact, in a recent contribution it has been observed an increased percentage of apoptosis in colon cancer cells transfected with a *KRAS* mutated vector, if compared to wild-type *KRAS* cells when both were treated with the same concentration of oxaliplatin *(Lin YL et al, 2012)*. Furthermore, it has been observed that *KRAS* mutations in colon cancer cells caused the down-regulation of ERCC1 (excision repair cross-complementation group 1), a protein involved in the mechanism of DNA damaged recognition and repair *(Lin YL et al, 2012)*. This might imply that some other unknown mechanisms could be responsible for the resistance to colon cancer treatment, in addition to the traditional RAS/RAF/MEK/ERK pathway.

1.7 BRAF

BRAF gene, located on chromosome 7, encodes for a RAS effector belonging to the RAF family of Ser-Thr kinase proteins. *BRAF* gene product is recruited to the plasma membrane upon binding to RAS-GTP, and represents a key point in the signal transduction through the MAP kinase pathway *(Figure 8)*.

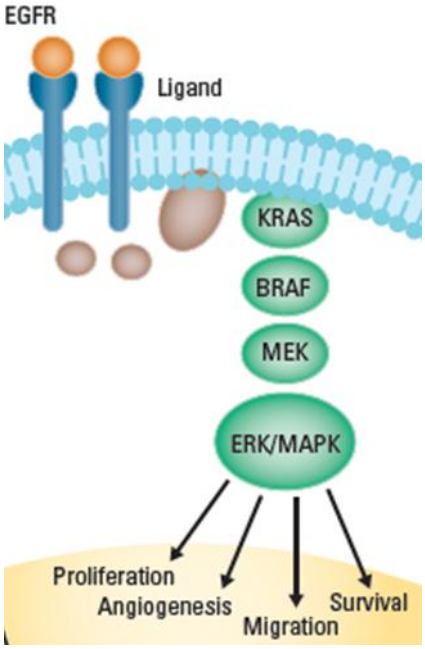

Figure 8: RAF and MAPK pathway (www.clarientinc.com).

BRAF sequence contains three conserved regions: CR1 encodes for the putative zinc finger domain, CR2, where several Ser-Thr-rich regions are located, and CR3, which corresponds to the kinase domain. The two major regulatory sites are Thr599 and Ser602, phosphorylated by RAS.

BRAF is the only RAF protein found to be frequently mutated in cancer. All mutations are represented by activating missense point mutations clustered in exons 11 and 15. In particular, the most common oncogenic *BRAF* mutation, occurring in more than 90% of cases, corresponds to a T>A transversion at position 1799 of *BRAF* sequence, resulting in the Valine to Glutamate substitution at position 600 of the protein (V600E) within the kinase domain, thus mimicking the phosphorylation of Thr599 and Ser602. This change leads therefore to a mutated BRAF protein with elevated kinase activity, able to constitutively activated MAPK pathway *(Davies H et al, 2002)*.

In colon cancer, *BRAF* mutations occur in about 10% of cases and are frequently found in sporadic cancers characterized by MSI *(Wang L et al, 2003)*. Recently, the correlation existing between *BRAF* mutations and MSI status was observed also in colon cancer patients who underwent FOLFOX-based adjuvant chemotherapy *(Sinicrope AF et al, 2013)*. In a recent study it has been observed that *BRAF* mutations were associated with a shorter survival after recurrence in FOLFOX treated patients and it could be considered a link with the MSI status. Tumors deficient in the MMR system showed a trend towards a poor survival after recurrence *(Gavin PG et al, 2012)*.

1.8 ERCC1

The product of *ERCC1* gene belongs to the nucleotide excision repair pathway, and it is required for repairing DNA lesions such as those induced by UV light or formed by electrophilic compounds including cisplatin. Nucleotide excision repair is a highly conserved DNA repair pathway that repairs DNA lesions which alter the helical structure of the DNA molecule and interfere with DNA replication and transcription. Important steps in this pathway include the recognition of DNA damage and demarcation of the specific area affected, followed by the formation of a complex to unwind the damaged portion and excise it. Finally, the excised area is resynthesized and ligated to maintain the DNA molecule. ERCC1 forms a heterodimer with the xeroderma pigmentosum (XPF) complementation group F (also known as ERCC4) and catalyzes the 5' incision in the process of excising the DNA lesion *(Figure 9)*.

The nucleotide excision repair system seems to be a key pathway involved in mediating resistance or sensitivity to platinum chemotherapeutic agents *(Martin LP et al, 2008)*.

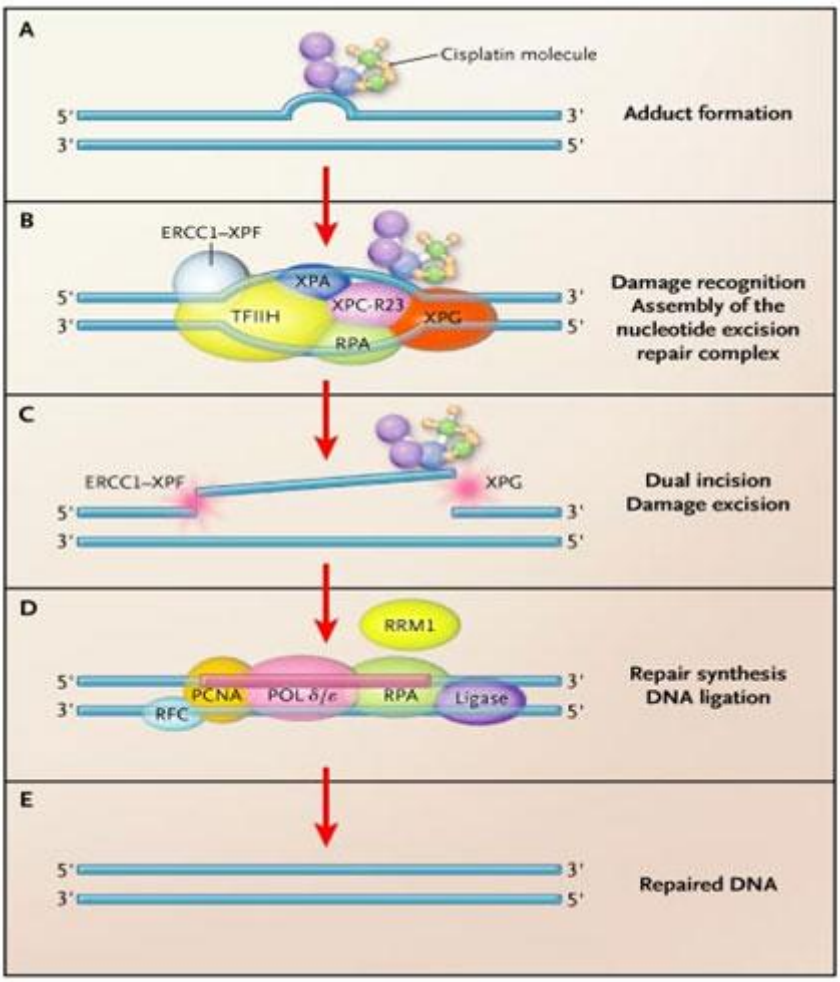

Figure 9: Mechanism of DNA damage recognition and repair (Fuss JO and Cooper PK, 2006).

ERCC1 complex in fact removes DNA adducts formed by platinum-based chemotherapies, leading to cancer cells survival. In this setting, ERCC1 can be considered as predictor of chemotherapy sensitivity. ERCC1 expression was investigated firstly in lung cancer, where platinum compounds are widely used. It has been observed that patients with low ERCC1 levels benefited from platinum-based chemotherapy in non-small cell lung cancer, whereas those with high levels did not *(Chen W and Bepler G, 2013)*. Also in gastric cancer, ERCC1 protein expression levels predict patients who more likely benefit from adjuvant cisplatin-based chemotherapy *(De Dosso S et al, 2013)*.

Regarding ERCC1 predictive role in colon cancer, it has been observed that in metastatic patients treated with oxaliplatin and 5FU chemotherapy, low ERCC1 expression correlated with a significantly longer median survival as compared to patients with high ERCC1 levels (median DFS: 10.2 and 1.9 months respectively),

nonetheless the association between ERCC1 levels and the response to chemotherapy did not reach a statistical significance (p=0.29) *(Shirota Y et al, 2001)*. Recently another study, investigating the role of ERCC1 in the adjuvant setting for stage III colon cancer patients, demonstrated that patients treated with oxaliplatin-based chemotherapy with positive ERCC1 tumors had a lower DFS (54%) and OS (60%) than those with negative ERCC1 tumors (72% and 78%, respectively) (p=0.009 for DFS values and p=0.02 for OS values). By contrast, ERCC1 status did not impact DFS (p=0.62) or OS (p=0.63) in 5FU group *(Li P et al, 2013)*.

Interestingly, in a recent study, preclinical data demonstrated that *KRAS* mutations in colon cancer cells caused ERCC1 down-regulation. KRAS knocking-down in *KRAS* mutated cancer cells led in fact to ERCC1 up-regulation and, at the same time, KRAS over-expression (mimic the effect of *KRAS* mutations) led to ERCC1 down-regulation and oxaliplatin sensitivity *(Lin YL et al, 2012)*. This significant finding might imply a possible correlation between *KRAS* mutations and ERCC1 expression.

In addition to ERCC1 expression, it has been proposed that also polymorphisms in *ERCC1* gene may influence response to treatment in colon cancer, at least in the metastatic setting. For instance, the analysis of the T19007C (codon 118) polymorphism revealed that the response rate to FOLFOX regimen was significantly higher in the TT genotype group compared to CT and CC groups (61.9%, 42.3%, and 21.4%, respectively, p=0.018). By contrast, no significant difference was observed when patients were treated with 5FU alone (45%, 29.2%, and 33.3%, respectively, p=0.407) *(Viguier J et al, 2005)*. However, other studies suggested that patients whose tumours were identified with TT genotype were associated with adverse DFS (p=0.02) *(Stoehlmacher J et al, 2004; Ruzzo A et al, 2007)*. Finally, another study suggested that CC genotype of *ERCC1* codon 118 polymorphism correlates with poor prognosis among patients receiving adjuvant chemotherapy *(Moreno V et al, 2006)*. Thus, the relationship between *ERCC1* codon 118 polymorphism and clinical outcome of patients receiving oxaliplatin-based chemotherapy for advanced colon cancer remains controversial.

1.9 TP53

Tumor protein 53, also known as *TP53*, is a tumor suppressor gene located on the short arm of chromosome 17. It is commonly considered the "guardian of the genome" for its critical function in suppressing tumorigenesis. *TP53* encodes for the tumor suppressor protein p53, which remains in the nucleus and performs its function there.

p53 deals with cells with damaged DNA by deciding the ultimate fates of these cells. It 'evaluates' whether the DNA damage is capable of being repaired; if so, p53 forms tetramers that are able to bind to the DNA and to activate the transcription of specific genes that can repair the damaged DNA. However, if the DNA is damaged beyond repair, p53 stops cells from replication and triggers the apoptosis of these cells. The function of p53 is not limited to the regulation of cells with damaged DNA. In fact, it is responsible for determining cell fate under other adverse conditions, such as lack of nucleotides for replication, hypoxia, and blockage of transcription *(Strachan T and Read AP, 1999) (Figure 10)*.

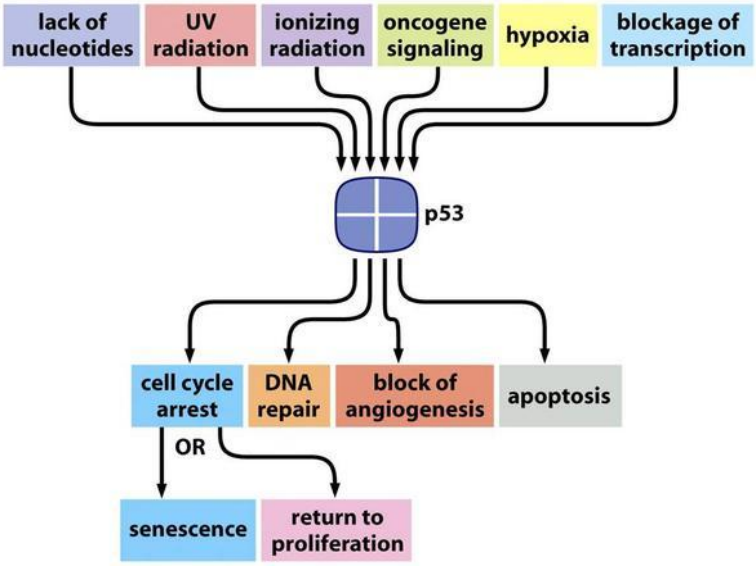

Figure 10: Functions of p53 (Weinberg R, *The Biology of Cancer*, 2007).

Loss of p53 function, which occurs through inactivating mutations or deletion of the two alleles, is one of the most common molecular events in colon cancer tumorigenesis, observed in nearly 50% of cases. As a consequence, several translational studies have evaluated its role in prognosis and response to therapy.

Overall, the abnormal immunohostochemical expression of p53 and *TP53* gene mutations are associated with increasead risk of death and a worse prognosis with respect to intact p53 status *(Chen J et al, 2013; Sarasqueta AF et al, 2013)*. Furthermore, p53 status was widely investigated because seemed to correlate with 5FU sensitivity, even if its role remains controversial. In stage III colon cancer patients, adjuvant therapy with 5FU improved 7-year survival in patients without p53 over-expression in respect with those patients exhibiting high p53 levels (64% versus 26%) *(Ahnen DJ, 1998)*. In another study, the presence of p53 mutation was not a predictive factor of OS ($p>0.05$) *(Elsaleh H et al, 2000)*, this result was also confirmed by another preclinical study on cell lines where no relation between *TP53* status and 5FU sensitivity at clinically relevant concentrations of 5FU was observed ($p>0.05$) *(Brody JR et al, 2009)*. Therefore, the role of p53 expression and *TP53* mutational status remains controversial in relation to 5FU adjuvant treatment.

Aim of the study

Nowadays, the gold treatment for advanced colon cancer (CC) is surgicalIy resection, followed by adjuvant chemotherapy. The standard option consists on the administration of 5-Fluorouracil (5FU) and folinic acid (FA) in combination with oxaliplatin (FOLFOX).

In spite of this, a proportion of patients will develop metastasis due to drug resistance, using a regimen that may cause potentially disabling sensory neuropathy. This happens because 5FU and oxaliplatin work by interfering with cellular functions. Oxaliplatin way of action, in particular, consists in binding to DNA and forming DNA adducts leading to cross-links which disrupt the structure of the DNA molecule and consequently run to steric changes in the helix. This alteration in the DNA structure leads to the activation of the cellular DNA damage recognition and repair system, which can result in the continued viability of the cell and, in the end, in treatment resistance.

Several proteins and genetic markers have been described in an attempt to refine prognostic information and predict the benefit derived from systemic treatment. In particular TS protein expression, MSI, p53 expression, *BRAF* and *TP53* mutations, have been described in several reports in relation to 5FU treatment, whereas *ERCC1* polymorphism, ERCC1 expression and *KRAS* mutations, seem to be related to oxaliplatin efficacy in advanced colon cancer patients *(Popat S et al, 2004; Brody JR et al, 2009; Gavin PG et al, 2012; Lin YL et al, 2012)*.

In order to better understand how such molecular markers could predict the clinical outcome in patients affected by colon cancer, we planned to analyze these markers in a cohort of surgically resected stage III CC patients who underwent FOLFOX adjuvant chemotherapy.

It is challenging to identify which patients are unlikely to benefit from adjuvant chemotherapy, because this would potentially spare many patients from acute and delayed drug-induced toxicities, and avoid the useless spending of public healthcare-resources.

Patients and Methods

3.1 PATIENTS

The study population consists of 230 patients from Switzerland and Argentina, recruited from 1998 to 2011 with curatively resected stage III colon cancer. After surgical resection, patients were treated with 6-months adjuvant chemotherapy, either with single-agent fluoropyrimidine (FP: modulated 5FU or capecitabine) or with oxaliplatin-based regimens (O-FP: FOLFOX or XELOX).

Drugs were administered as follows:

- 5FU: 5-fluorouracil 370-420 mg/m^2 plus leucovorin 20-200 mg/m^2 on days 1-5, repeated every 4-5 weeks for 6 months ;
- Capecitabine: 1000–1250 mg/m2 twice-daily on days 1-14, repeated every 3 weeks for 6 months;
- FOLFOX: a two-hours infusion of leucovorin 200 mg/m^2 followed by a 400 mg/m^2 bolus 5FU followed by 22-hours infusion of 5FU 600 mg/m^2 given on two consecutive days plus a two-hours infusion of 85 mg/m^2 oxaliplatin, on day 1, simultaneously with leucovorin, using a Y-infusion device;
- XELOX: oxaliplatin 130 mg/m^2 IV infusion over two hours (day 1 every three weeks) in combination with capecitabine administrated orally at dose of 1000 mg/m^2 twice-daily (equivalent to a total daily dose of 2000 mg/m^2, the first evening dose on day 1 and the last morning dose on day 15) given as intermittent treatment (three-weeks cycles consisting of two weeks of treatment followed by one week without treatment).

3.2 CLINICAL EVALUATION

The clinical outcome was monitored for each patient from surgery to death or to last follow-up date.

After surgery and adjuvant chemotherapy, post-treatment surveillance of patients was performed to evaluate possible therapeutic complications, discover a recurrence that was potentially resectable for cure, and to identify new metachronous neoplasms at a pre-invasive stage.

The surveillance during follow-up period consisted on history and physical examination every 3 to 6 months for 2 years, then every 6 months for 3 years and

then once a year; Carcino-Embryonic Antigen (CEA) test was evaluated at baseline and in combination with scheduled physical controls. Colonoscopy was accomplished at approximately 1 year after resection (or approximately 3–6 months post-resection if not performed preoperatively due to obstructing lesion), than once a year until 5 years after resection and every two years thereafter. More frequent colonoscopies were indicated in patients who presented a colon cancer before age 50. Chest, abdominal, and pelvic computed tomography scans were performed annually for the first 3 to 5 years, and every two years thereafter. Adverse chemotherapy events, such as chronic diarrhoea or incontinence or persistent neuropathy (a well known side effect of oxaliplatin treatment) were monitored in patients' follow-up period.

We considered the DFS as the time from the date of surgery to the date of relapse or other malignancies occurrence or death, whichever came first. Patients alive and free of relapse or other malignancies were censored at the time of last follow-up date. OS was defined as the period existing between surgery resection and patients' death or last follow-up date.

3.3 MOLECULAR ANALYSIS

All the analyses were performed on formalin-fixed paraffin-embedded (FFPE) tissue sections. For immunohistochemical and molecular analyses, a single representative FFPE tumor tissue block, containing at least 70% of neoplastic cells was selected for each sample *(Van Krieken JH et al, 2008)*. Argentinean cases were fixed in Bouin and then embedded in paraffin. Tumour macrodissection was performed in tumour blocks containing less than 70% of neoplastic cells to reduce the presence of non-neoplastic tissues. For microsatellite and RNA analyses, a paired healthy mucosa tissue block was also chosen.

DNA extraction was performed using the QIAamp Mini kit (Qiagen, Chatsworth, CA, USA) while the RNeasy FFPE Kit (Qiagen) was used for RNA extraction, according to the manufacturer's instructions.

3.4 MSI

The status of MSI was assessed by the analysis of the microsatellite loci included in the panel of Bethesda (BAT 25, BAT 26, D2S123, D5S346 AND D17S250), as reported in the literature *(Frattini M et al, 2004a)*. MSI was confirmed by the presence of additional peak(s) in tumour sample compared with the normal paired tissue. MSI was defined as being present when more than 30% of investigated loci showed instability.

3.5 KRAS, BRAF, ERCC1 AND TP53 MUTATIONAL ANALYSIS BY DIRECT SEQUENCING

KRAS, *BRAF*, *ERCC1* and *TP53* mutational status was assessed by direct sequencing on genomic DNA, as already reported *(Frattini M et al, 2004a; Frattini M et al, 2004b; Stoehlmacher J et al, 2004; Liu D et al, 2005)*.

In particular we investigated:

- *KRAS* exon 2, including hot spots in codons 12 and 13;
- *BRAF* exon 15, including hot spot in codon 600;
- *ERCC1* exon 4, including the polymorphism at codon 118;
- *TP53* exons 5, 6, 7 and 8, including hot spots in codons 175, 248, 273 and 282.

In the investigated exons of *KRAS*, *BRAF* and *TP53* genes, more than 80-90% of mutations occur *(Davies H et al, 2002; Samowitz WS et al, 2002; Frattini M et al, 2004a; Stoehlmacher J et al, 2004; www.sanger.ac.uk/genetics/CGP/cosmic/)*.

Genomic DNA was amplified by Polimerase Chain reaction (PCR), purified (MSB®SPIN PCRapace, STRATEC Molecular, Gmbh, Berlino, Germania) and directly sequenced. The list of primers used is reported in Table 1.

The direct sequencing analysis was performed by ABI Prism 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA) as previously described and the Sequencing Analysis software was used for data evaluation *(Frattini M et al, 2007)*. Each sequence reaction was carried out at least twice, starting from independent PCR reactions. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands.

Gene	Exon	Forward Primer	Reverse Primer	Annealing T
KRAS	2	TGGTGGAGTATTTGATAGTGTA	CATGAAAATGGTCAGAGAA	55°C
BRAF	15	TCATAATGCTTGCTCTGATAGGA	GGCCAAAAATTTAATCAGTGGA	52°C
ERCC1	4	GCAGAGCTCACCTGAGGAAC	GAGGTGCAAGAAGAGGTGGA	65°C
TP53	5	TTCAACTCTGTCTCCTTCCT	CAGCCCTGTCGTCTCTCCAG	62°C
TP53	6	GCCTCTGATTCCTCACTGAT	TTAACCCCTCCTCCCAGAGA	62°C
TP53	7	AGGCGCACTGGCCTCATCTT	TGTGCAGGGTGGCAAGTGGC	64°C
TP53	8	TTCCTTACTGCCTCTTTGCTT	AAGTGAATCTGAGGCATAAC	56°C

Table 1: Primers used for PCR reactions.

3.6 ERCC1 IMMUNOHISTOCHEMICAL ANALYSIS

The immunohistochemical (IHC) analysis was performed on 3-µm thick FFPE tissue section by using anti-ERCC1 (clone 8F1, dilution 1:50; ThermoScientific, Erembodegem, Belgium) monoclonal antibody. The analysis was performed on Ventana BENCHMARK® XT instrument using UltraView DAB kit (Ventana Medical Systems, Tucson, USA). Briefly, for epitope retrieval, slides were exposed on heat EDTA, then endogenous peroxidase activity was blocked by incubation with H_2O_2 3% (30 min EDTA and 4 min H_2O_2). Primary antibody incubation was carried out for 32 minutes at 37°C. Immunoreaction was revealed by secondary antibody incubation for 8 min with 3'-3'-diaminobenzidine as the chromogen, and Mayers hematoxylin as the counterstain. Endothelial cells of normal tonsil tissues and proliferating germinal centre lymphocytes were included as positive controls for ERCC1, as previously suggested *(Olaussen KA et al, 2006; Zucali PA et al, 2011)*.

Immunostaining was evaluated under a light microscope by an expert pathologist (Prof. Renzo Boldorini, Deparment of Medical Sciences, University of Eastern Piedmont, Italy). A positive staining was assigned when tumour cells showed nuclear reactivity. In details, the intensity of staining was scored on a scale of 0 to 3; with 3 indicating the higher intensity using normal tonsil tissue as positive control. Then we looked at the percentage of positive tumour cells, scoring as follow: 0 if 0%; 0.1 if 1% to 9%; 0.5 if 10% to 49%; 1 if 50% or more. Finally, since to date there are no standardized guidelines for ERCC1 staining evaluation on colon tumours, an H-score usually utilized in the evaluation of ERCC1 in non-small cell lung cancer was applied *(Olaussen KA et al, 2006)*. Semiquantitative H-score was obtained from intensity multiplied with positive cells, with values ranging from 0 to 3.

The median value of all H-score was chosen as the cut off point to determine positive or negative tissues according to the literature *(Olaussen KA et al, 2006; Zucali PA et al, 2011)* and to our previous work on gastric cancer *(De Dosso S et al, 2013)*.

3.7 ERCC1 ANALYSIS BY REAL-TIME PCR

To analyze *ERCC1* gene expression, a fluorescence-based real-time procedure (StepOne™ Real-Time PCR System; Applied Biosystems) was adopted, using the protocol previously described *(Gibson UE et al, 1996; Heid CA et al, 1996; Sørby LA et al, 2010; Kheirelseid EA et al, 2010)*. *Pol2RA* was chosen as internal reference gene.

Total RNA was transcribed into cDNA using the High Capacity RNA-to-cDNA™ Master Mix protocol (Applied Biosystems). Real Time PCR was performed in the CFX96™ Real Time System (Bio-Rad, Hercules, CA, USA). Taqman® Gene Expression Master Mix and Taqman® assays probes (*POL2RA* probe: Hs00172187_m1 and *ERCC1* probe: Hs01012161_m1) were purchased from Applied Biosystems.

Each sample was analyzed in triplicate. Final results were determined by the formula $2^{-\Delta\Delta Ct}$, which standardizes the target with the reference gene in both tumour and normal tissue *(Livak KJ and Schmittgen TD, 2001)*. The median value of all scores was used as threshold line separating ERCC1 overexpressing (higher values) from ERCC1 normally expressing (lower values) cases.

3.8 STATISTICAL ANALYSES

A two-tailed Fisher's exact test was used to calculate the p values for the association among variables. Level of significance was set at p=0.05. The DFS and OS analyses were performed according to the Kaplan–Meier method, and survival curves were compared using the log-rank test. Data were analyzed using the IBM SPSS Statistics 20 package.

Results

4.1 PATIENTS CHARACTERISTICS

Two-hundred and thirty patients with advanced colon cancer (stage III), were enrolled in this study. The population included 103 women and 127 men. The median age at diagnosis was 62 years (range: 22-82). One-hundred and six patients received 5FU as single-agent, whereas the remaining 124 received FOLFOX. The presence of relapse was observed in 77 (33%) cases, 39 of whom were treated with only fluoropyrimidine and 38 received also the oxaliplatin. All patients' characteristics are reported in Table 2. The median DFS and OS follow-up period were 45.9 months (range: 1.7-177 months) and 52.4 (range: 2.5-177 months) respectively.

Patients characteristics (N=230)	Number of cases	Percentage (%)
Age		
> 65	96	42
≤ 65	134	58
Gender		
male	127	55
female	103	45
Treatment		
5FU	106	46
FOLFOX	124	54
Relapse		
yes	77	33
no	153	67

Table 2: Patients' clinical-pathological characteristics.

Age and gender were correlated with the DSF and OS for the entire cohort, but no statistical significance was observed.

By stratifying the population for the two treatments, we found that patients who received FOLFOX (median DFS and OS: 51.3 and 55.4 months, respectively) seem to get more benefit by chemotherapeutic treatment if compared to patients treated with 5FU alone (median DFS and OS: 41.6 and 49.3 months, respectively), even if those results did not reach a statistically significant value, in both DFS (p=0.54) *(Figure 11A)* and OS (p=0.22) *(Figure 11B)*.

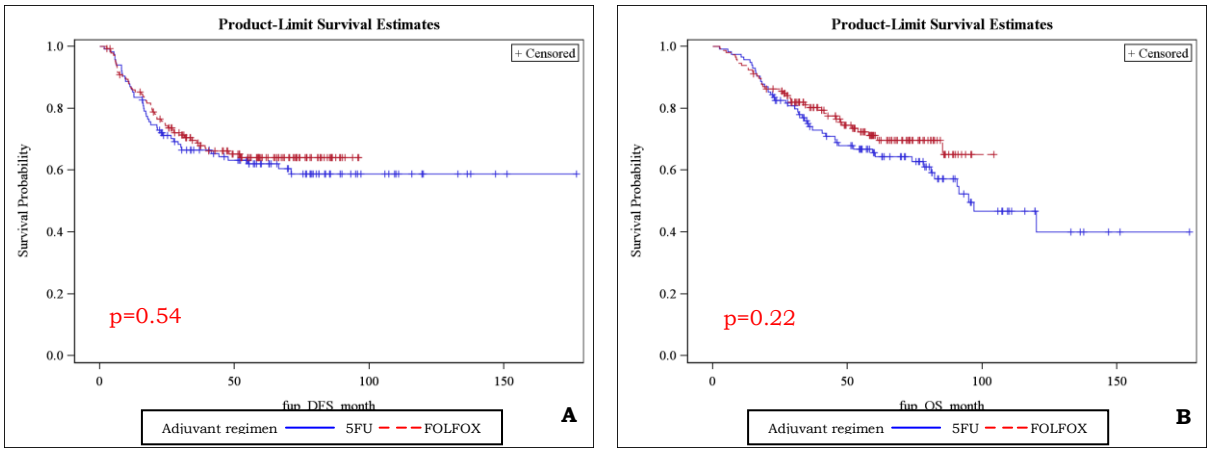

Figure 11: DFS (A) and OS (B) for the entire population stratified for the received treatment. 5FU= 5-fluorouracil; FOLFOX= 5-fluorouracil plus oxaliplatin.

4.2 MOLECULAR ANALYSES

MSI, *KRAS*, *BRAF*, *ERCC1* and *TP53* molecular results are summarized in Table 3. Every molecular marker was correlated with the survival probability, in order to verify if a prognostic value could be associated to each one.

Marker (N)	Molecular alterations	Number of alterations	Percentage (%)
MSI (N=209)	MSI	26	12
	MSS	183	88
KRAS (N=229)	Mutated	65	28
	Wild-type	164	72
BRAF (N=224)	Mutated	21	9
	Wild-type	203	91
ERCC1 (N=221)	TT genotype	69	31
	CT genotype	105	48
	CC genotype	47	21
TP53 (N=189)	Mutated	83	44
	Wild-type	106	56

Table 3: Molecular results. N = number of cases evaluable for a given marker.

4.2.1 MSI

Microsatellite status was assessed in 209 cases, because 21 were not evaluable. The results showed MSS in 183 cases (88%) whereas MSI was detected in the remaining 26 cases (12%) *(Table 3)*.

The correlation with the DFS (p=0.17) revealed a better prognostic trend for patients showing MSI if compared to those with MSS, as reported in the Kaplan Meyer curve

(Figure 12A), even if this result did not reach a statistical significance. On the contrary, the correlation with the OS (p=0.48) was not statistical significant *(Figure 12B)*.

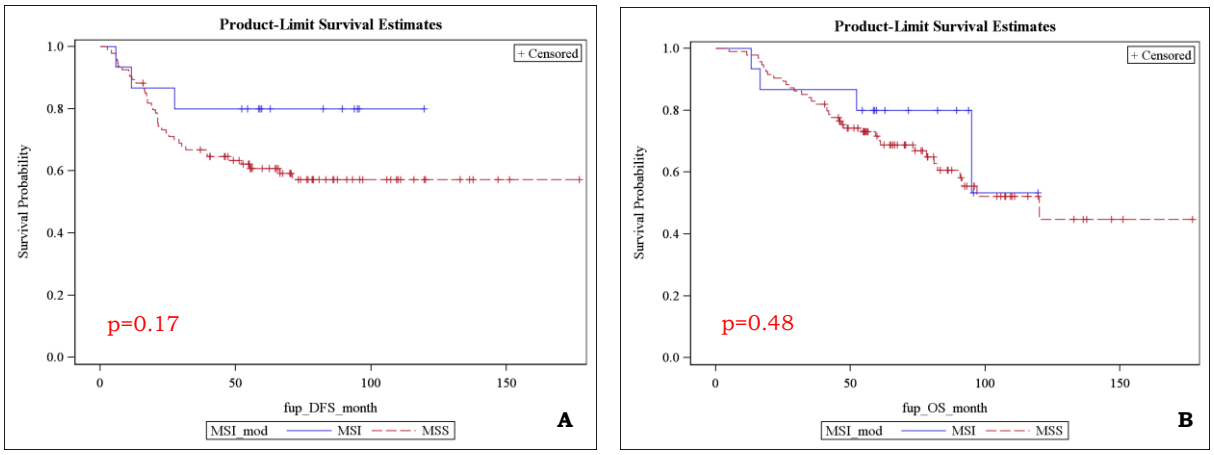

Figure 12: DFS (A) and OS (B) for MSI and MSS patients.

4.2.2 *KRAS*

The characterization of *KRAS* mutational status was not possible in 1 case. We identified 65 (28%) mutations, 52 in codon 12 and 13 in codon 13 *(Table 3)*. In particular, in codon 12 we detected the following alterations: G12D in 23 cases, G12V in 13 cases, G12A in 6 cases, G12C in 5 cases, G12S in 4 cases and G12R just in 1 case. Regarding codon 13, we identified the G13D alteration in all the 13 cases.

By correlating *KRAS* status with clinical follow-up, *KRAS* mutations confer a worse prognosis to advanced colon cancer patients, borderline for the DFS (p=0.07) *(Figure 13A)* and statistically significant for the OS (p=0.004) *(Figure 13B)*.

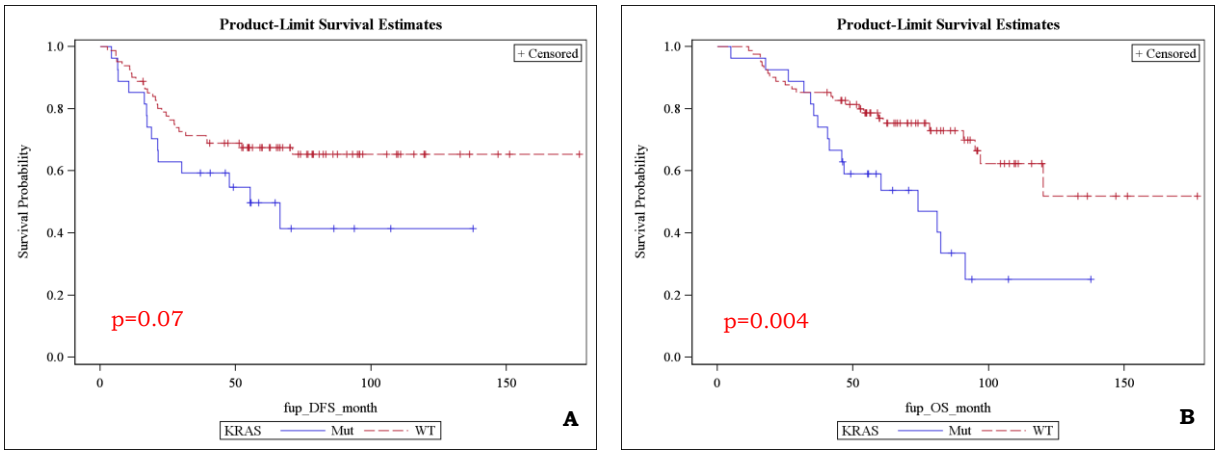

Figure 13: DFS (A) and OS (B) for *KRAS* mutated (Mut) and wild-type (WT) patients.

4.2.3 *BRAF*

We performed *BRAF* mutational analysis in 224 cases, because 6 cases were not evaluable. We detected 21 alterations (9%) *(Table 3)*, in all cases the mutation identified was the classical V600E change.

The correlation between *BRAF* mutational status and clinical follow-up was not statistical significant, for both DFS (p=0.40) *(Figure 14A)* and OS (p=0.53) *(Figure 14B)*.

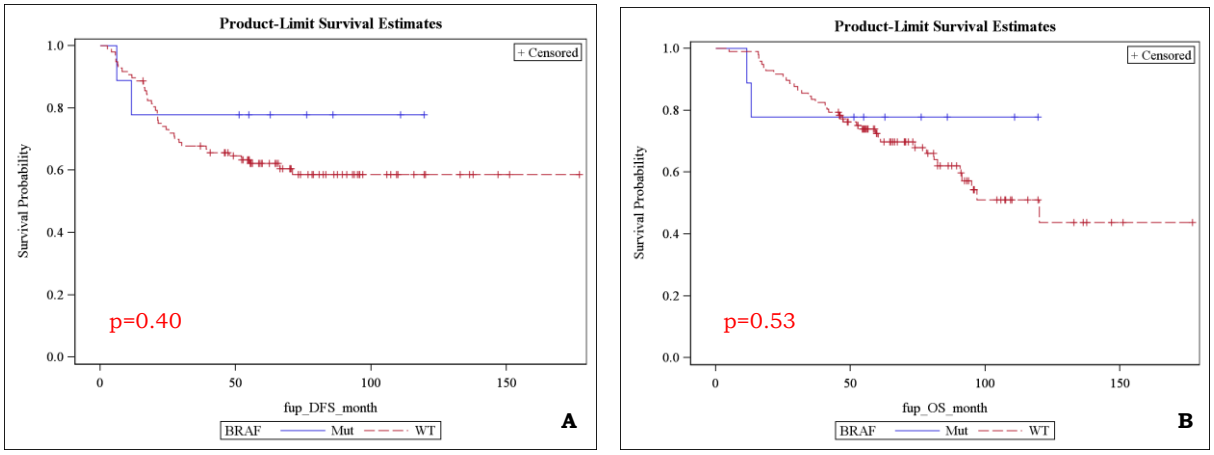

Figure 14: DFS (A) and OS (B) for *BRAF* mutated (Mut) and wild-type (WT) patients.

4.2.4 *ERCC1*

The characterization of codon 118 (AAT/AAC) polymorphism was possible in 221 cases. We identified TT genotype in 69 patients (31%), CT genotype in 105 patients (48%) and the CC variant in 47 cases (21%) *(Table 3)*. Therefore, the T allele was present in 79% of cases and the C allele in 69% of patients. The correlation with the clinical data is on-going.

4.2.5 *TP53*

TP53 analysis was performed in 189 cases as 41 were classified as not evaluable. Overall, we identified 83 mutations (44%) *(Table 3)*. In particular, we detected the vast majority of alterations in exons 5 and 7. Seventy-seven cases displayed one mutation, whereas 6 patients carried two alterations. The most frequent identified mutations were R175H (in exon 5) in 8 cases and G245S, R248Q, R248W (all changes in exon 7) and R306X (in exon 8) in 4 cases each *(Table 4)*. The presence of a frameshift mutation (due to a deletion or an insertion of one or more nucleotides) was detected in 11

cases, in particular: 2 in exon 5, 2 in exon 6, 4 in exon 7 and 3 in exon 8. The remaining mutations identified are reported below *(Table 4)*.

The correlation with the clinical follow-up is on-going.

TP53			
exon 5	**exon 6**	**exon 7**	**exon 8**
R152S	R196X	Y233X	G266R
R158L	R213X (2)	Y234H	E271K
A159V	H214R	Y236C	R273C (2)
A161T	V216M	M237I	R273H
Y163C	V220C	M237V	V274F (2)
Y163N	Y220H	C238Y	V274L
Q165X	V225G	N239D	P278L,
E171X	FS (2)	N239S	R280K
V172F		C242F	D281Y
R174W		G244D	R282W (2)
R175H (8)		G245C (2)	R283H
C176G		G245D (2)	E285K
C176R		G245S (4)	K305M
H178Y		R248Q (4)	R306X (4)
H179L		R248W (4)	FS (3)
H179Y		FS (4)	
FS (2)			

Table 4: List of *TP53* mutations identified in our cohort, the numbers in brackets correspond to the number of patients with that alteration. FS = frameshift.

4.3 ERCC1 EXPRESSION

ERCC1 expression levels were analyzed both by IHC and by real-time PCR. The results are reported in Table 5.

ERCC1 detection	Positive cases	Percentage
IHC	88/218	40%
Real-Time PCR	102/207	49%

Table 5: ERCC1 positive expression levels detected by IHC and by real-time PCR.

4.3.1 IHC

The ERCC1 protein expression analysis, studied by IHC, was successful in 218 cases as 12 cases were not evaluable. At first, we evaluated for each patient the intensity of staining, on the basis of pathologist's evaluation: 79 were classified as 0, 64 as 1+, 46 as 2+ and 29 as 3+. Then we considered the percentage of cells recognized as positive

to ERCC1 staining (from 0% to 100%). By combining the two scores we obtained H-values, ranging from 0.1 to 3. On the basis of the literature, assuming the median H-score value (0.5 in our cohort) as the cut-off point, 88 (40%) were considered positive (>0.5) *(Figure 15A)* and 130 (60%) were classified as negative (≤0.5) for ERCC1 protein expression *(Figure 15B) (Table 5)*.

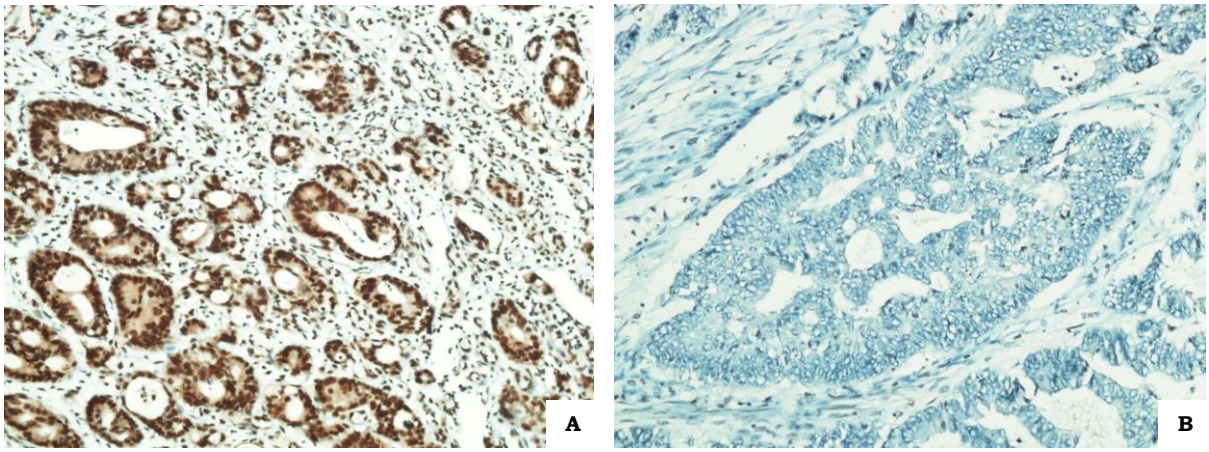

Figure 15: Examples of ERCC1 protein expression, positive, score 3 (A) and negative, score 0 (B).

When we correlated the clinical follow-up with IHC data, we classified the cases on the basis of two parameters: in the first one we considered ERCC1 negative cases only those with ERCC1 score 0 (and then we performed the correlation with the group of score 1+, score 2+, score 3+), in the second one we considered as negative those cases with score 0 and 1+. The results were not statistically significant in both situations. Finally, we correlated the clinical follow-up with the median H-score (0.5), and also with these parameters we did not find any statistical difference (p=0.40 for DFS and p=0.55 for OS) *(Figure 16A,B)*.

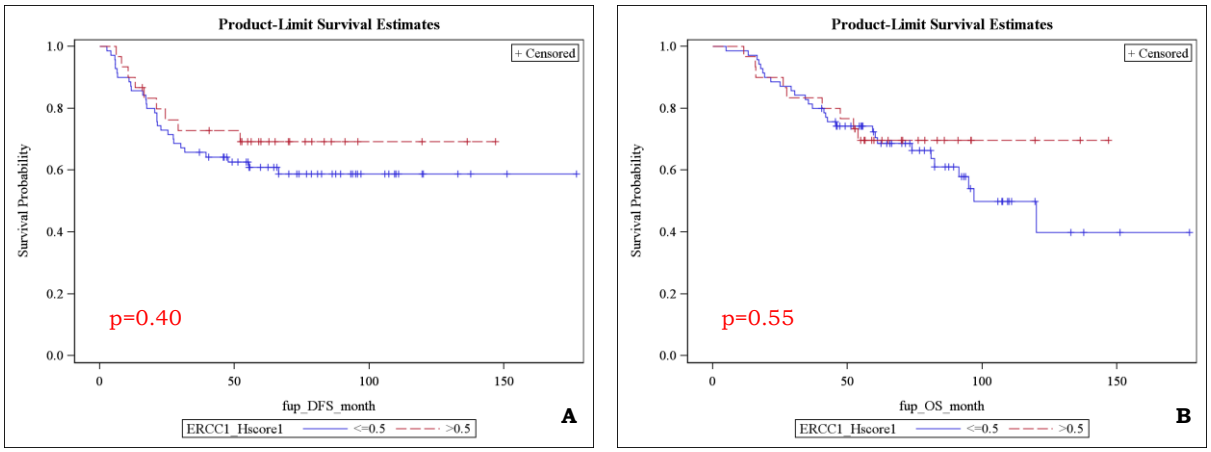

Figure 16: DFS (A) and OS (B) for patients exhibiting low (≤0.5) and high (>0.5) ERCC1 protein expression levels.

4.3.2 REAL-TIME PCR

The analysis of ERCC1 mRNA expression was successful in 207 patients. Values ranged from 0.2 to 16.8, with a median value of 1.20. According to the literature, using the median value as the cut-off point, in our cohort 102 (49%) were considered overexpressing ERCC1 (values higher than 1.20), whereas in 105 (51%) cases ERCC1 resulted as down-regulated *(Table 5)*. By correlating the real-time results with the clinical follow-up, we observed a trend toward a better DFS (p=0.11) for those patients showing lower ERCC1 mRNA levels *(Figure 17A)* and there was no difference in the OS (p=0.37) for patients characterized by ERCC1 down- or up- regulation *(Figure 17B)*.

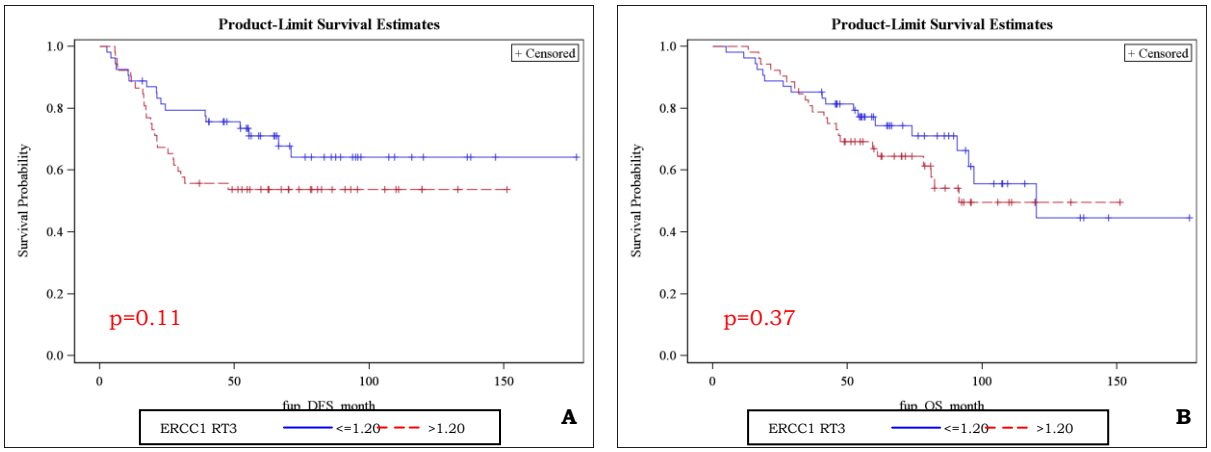

Figure 17: DFS (A) and OS (B) for patients exhibiting low (≤1.20) and high (>1.20) ERCC1 mRNA levels.

4.3.3 CORRELATION BETWEEN ERCC1 PROTEIN AND mRNA LEVELS

When we correlated the results of ERCC1 protein expression by IHC with those of ERCC1 mRNA expression by Real-Time PCR, we did not find any association, also taking into account all the possible immunohistochemical classifications.

4.4 MOLECULAR MARKERS CORRELATIONS

In our cohort, we can confirm the mutual exclusivity existing between *KRAS* and *BRAF* mutations: in fact, all 65 *KRAS* mutated cases were *BRAF* wild-type, and all 9 *BRAF* mutated cases showed a *KRAS* wild-type sequence.

By correlating microsatellite status and *BRAF* mutations, we were able to identify a statistically significant association between the two molecular markers (p=0.0052, two-tailed Fisher's exact test) *(Table 6)*.

	BRAF mutated	BRAF wild-type	p value
MSI	8	18	p=0.0052
MSS	9	168	

Table 6: Microsatellite pattern and *BRAF* mutational status association (two-tailed Fisher's exact test).

No association was found between *KRAS* mutations and ERCC1 expression, in both protein (p=0.28) *(Table 7)* and mRNA (p>0.99) *(Table 8)* levels.

	KRAS mutated	KRAS wild-type	p value
IHC>0.5	21	67	p=0.28
IHC≤0.5	40	89	

Table 7: ERCC1 protein expression (detected by IHC, using H-score classification) and KRAS mutational status association (two-tailed Fisher's exact test).

	KRAS mutated	KRAS wild-type	p value
RT>1.20	31	70	p>0.99
RT≤1.20	32	73	

Table 8: ERCC1 mRNA levels (detected by real-time PCR) and *KRAS* mutational status association (two-tailed Fisher's exact test).

4.5 MOLECULAR MARKERS AND CLINICAL OUTCOME

The molecular results for the entire cohort were then correlated with the clinical outcome of patients treated with the two different modalities, 5FU alone or in addition to oxaliplatin (FOLFOX). The detailed correlations are reported below.

4.5.1 MSI and treatment

We identified MSI in 12 (13%) patients treated with 5FU and in 14 (12%) treated with FOLFOX. By correlating microsatellite status and 5FU treatment, the result we obtained was not statistical significant for DFS (p=0.16) *(Figure 18A)* and OS (p=0.37) *(Figure 18B)*, but in the former a trend toward a better outcome was found in patients showing MSI.

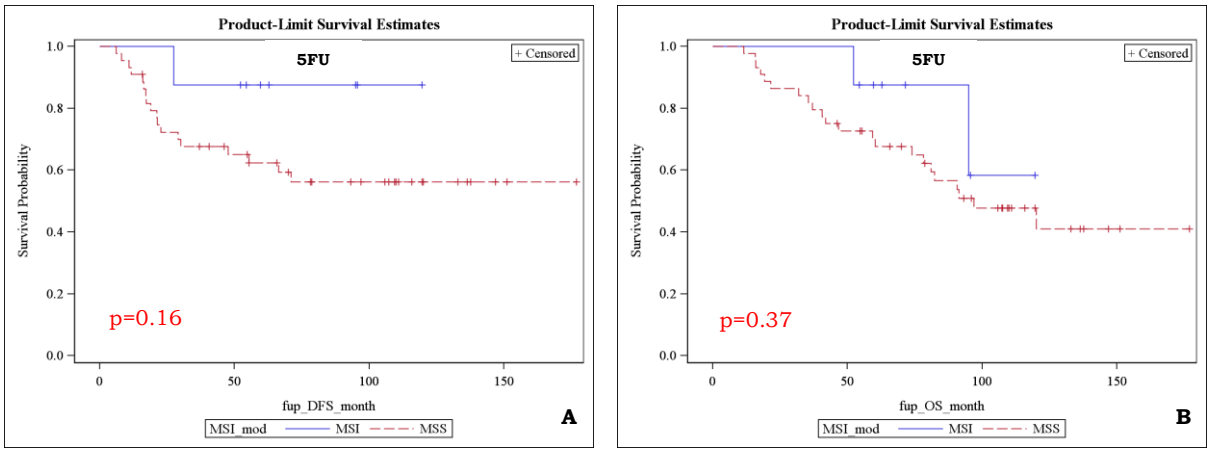

Figure 18: DFS (A) and OS (B) for MSI and MSS in 5FU treated patients.

By correlating microsatellite status and FOLFOX treatment, there was no difference in the clinical outcome, for both DFS (p=0.67) *(Figure 19A)* and OS (p=0.86) *(Figure 19B)* for patients exhibiting MSI or MSS.

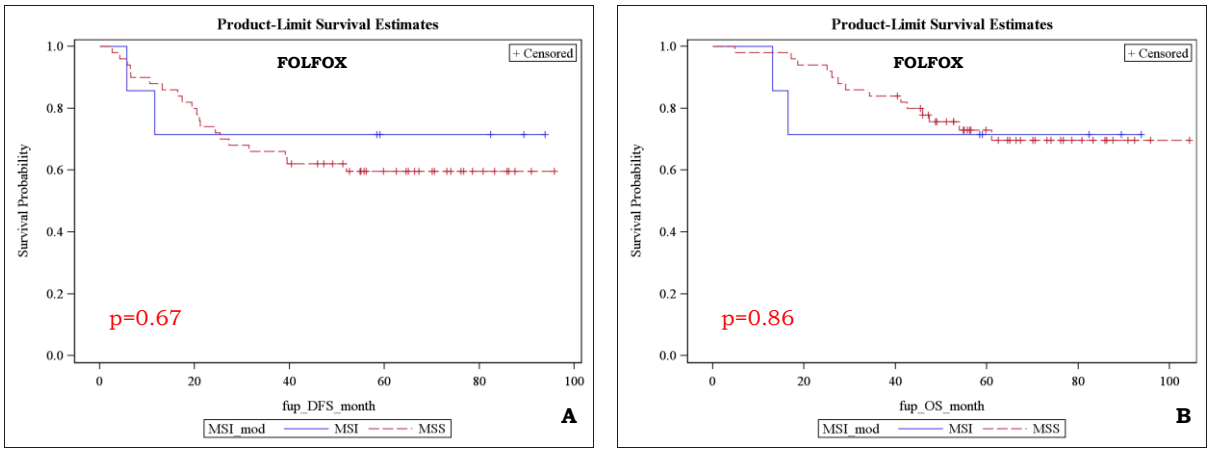

Figure 19: DFS (A) and OS (B) for MSI and MSS in FOLFOX treated patients.

4.5.2 *KRAS* and treatment

Above all, we identified 31 (30%) *KRAS* mutations in the 5FU group and 34 (27%) in FOLFOX cases.

By correlating *KRAS* mutational status with the clinical outcome of patients treated with 5FU, a statistical significant worse DFS (p=0.04) *(Figure 20A)* and a trend toward a worse OS (p=0.07) *(Figure 20B)* were observed in *KRAS* mutated patients. The median DFS and OS were 46.0 and 56.1 months, respectively, in *KRAS* mutated patients, and 57.1 and 62.0 months, respectively, in *KRAS* wild-type cases.

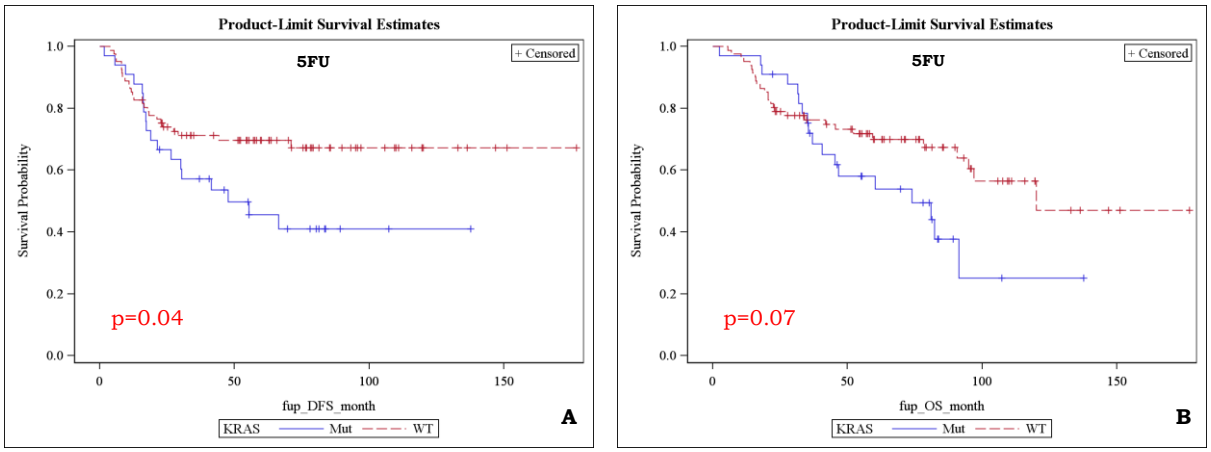

Figure 20: DFS (A) and OS (B) for *KRAS* mutated (Mut) and wild-type (WT) patients treated with 5FU.

Then, we correlated *KRAS* gene status with the outcome of patients who were treated with FOLFOX regimen.

DFS (p=0.62) *(Figure 21A)* and OS (p=0.77) *(Figure 21B)* were not statistically different for mutated and wild-type *KRAS* patients treated with FOLFOX: the median DFS and

OS were 43.7 and 48.9 months, respectively, in the mutated group, and 44.4 and 49.6 months, respectively, in wild-type patients.

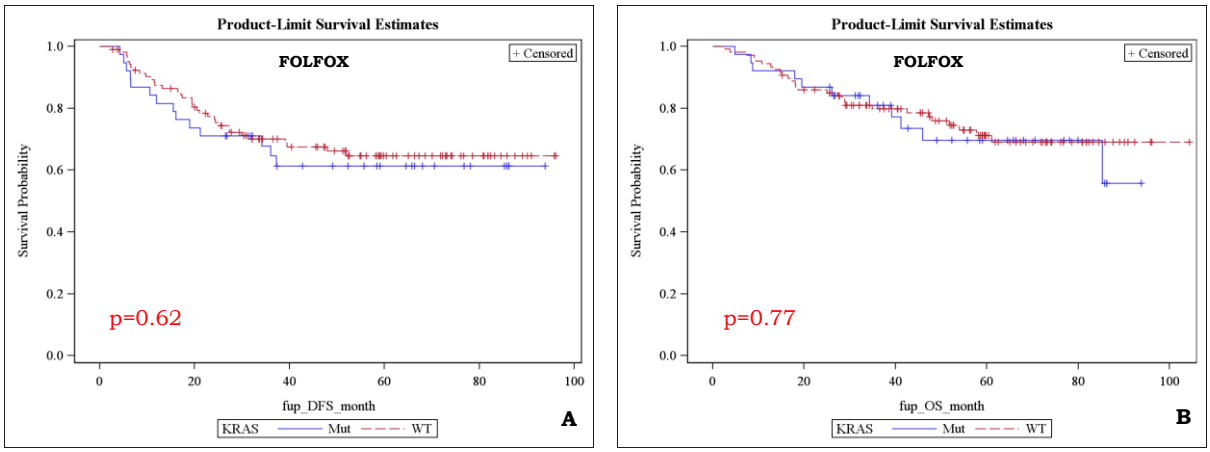

Figure 21: DFS (A) and OS (B) for *KRAS* mutated (Mut) and wild-type (WT) patients treated with FOLFOX.

Stratifying the population on the basis of *KRAS* mutational status, we observed that in wild-type *KRAS* cases, DFS (p=0.78) *(Figure 22A)* and OS (p=0.68) *(Figure 22B)* did not significantly differ between the two treatment modalities: the median DFS and OS were 44.4 and 49.6 months, respectively, in FOLFOX group, and 57.1 and 62.0 months, respectively, for the 5FU regimen.

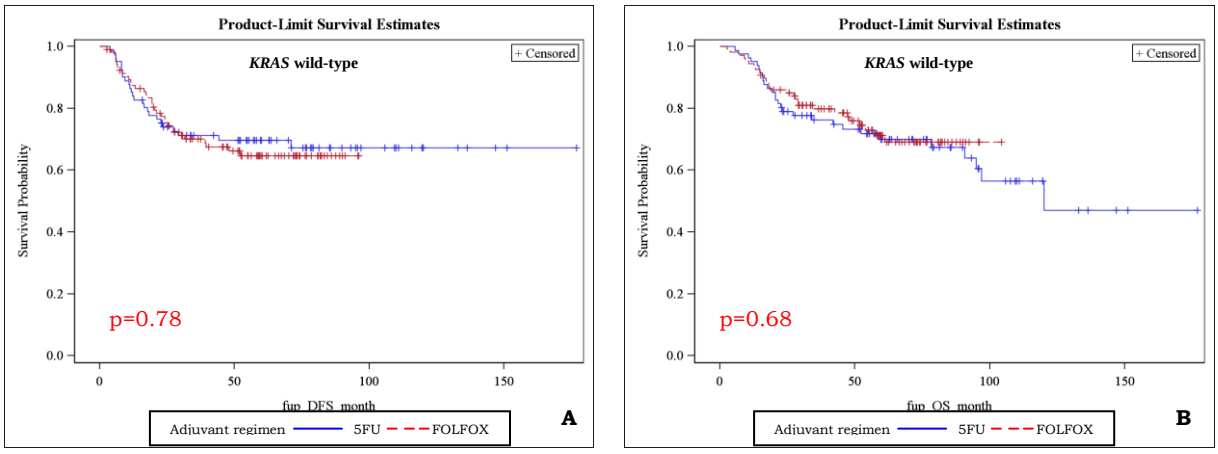

Figure 22: DFS (A) and OS (B) for *KRAS* wild-type cases stratified for the received treatment. 5FU= 5-fluorouracil; FOLFOX= 5-fluorouracil plus oxaliplatin.

In *KRAS* mutated cases, a trend toward better DFS (p=0.28) *(Figure 23A)* and OS (p=0.20) *(Figure 23B)* was observed in FOLFOX treated patients with respect to 5FU

group, although without reaching statistical significance. The median DFS and OS were 43.7 and 48.9 months, respectively, in FOLFOX group, and 46.0 and 56.1 months, respectively, in 5FU group.

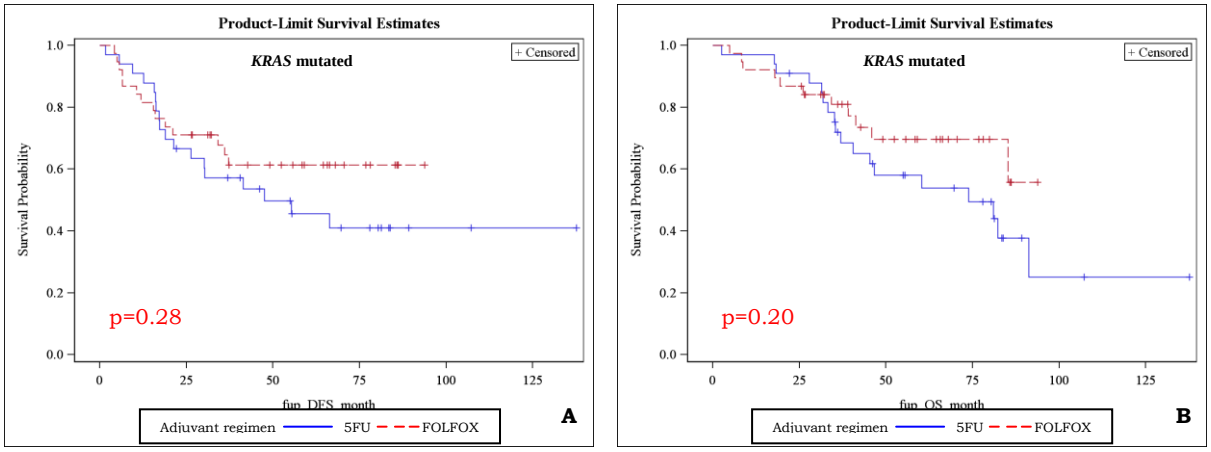

Figure 23: DFS (A) and OS (B) for *KRAS* mutated cases stratified for the received treatment. 5FU= 5-fluorouracil; FOLFOX= 5-fluorouracil plus oxaliplatin.

4.5.3 *BRAF* and treatment

We identified 9 (8.6%) *BRAF* mutations in the 5FU group and 12 (10%) in the FOLFOX cases. We correlated *BRAF* mutational status in both 5FU and FOLFOX group. Our results did not show statistical difference in the clinical outcome for mutated or wild-type *BRAF* patients in 5FU regimen, for both DFS (p=0.70) *(Figure 24A)* and OS (p=0.55) *(Figure 24B)*.

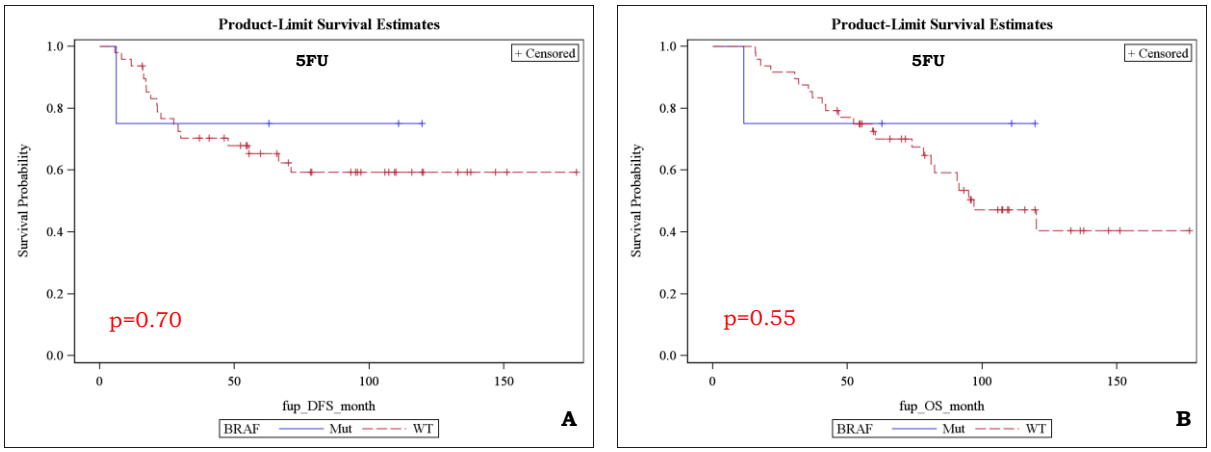

Figure 24: DFS (A) and OS (B) for *BRAF* mutated (Mut) and wild-type (WT) patients, treated with 5FU.

Also in FOLFOX group, DFS (p=0.43) *(Figure 25A)* and OS (p=0.78) *(Figure 25B)* were not statistical different for *BRAF* mutated or wild-type patients.

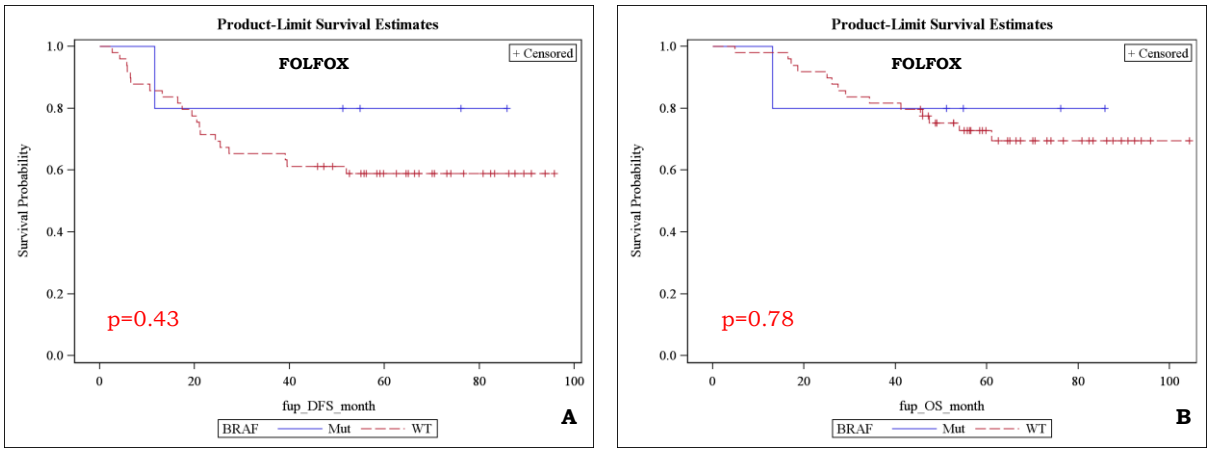

Figure 25: DFS (A) and OS (B) for *BRAF* mutated (Mut) and wild-type (WT) in FOLFOX treated patients.

4.5.4 ERCC1 and treatment

Looking at immunohistochemical results, taking into account the H-score, we identified 64 (66%) patients in the 5FU group and 66 (55%) in the FOLFOX group showing a down-regulation of ERCC1 expression levels (H score ≤0.5). By correlating ERCC1 protein expression levels with 5FU patients' clinical outcome, no statistical difference was identified between ERCC1 up- or down- regulation in both DFS (p=0.47) *(Figure 26A)* and OS (p=0.89) *(Figure 26B)*.

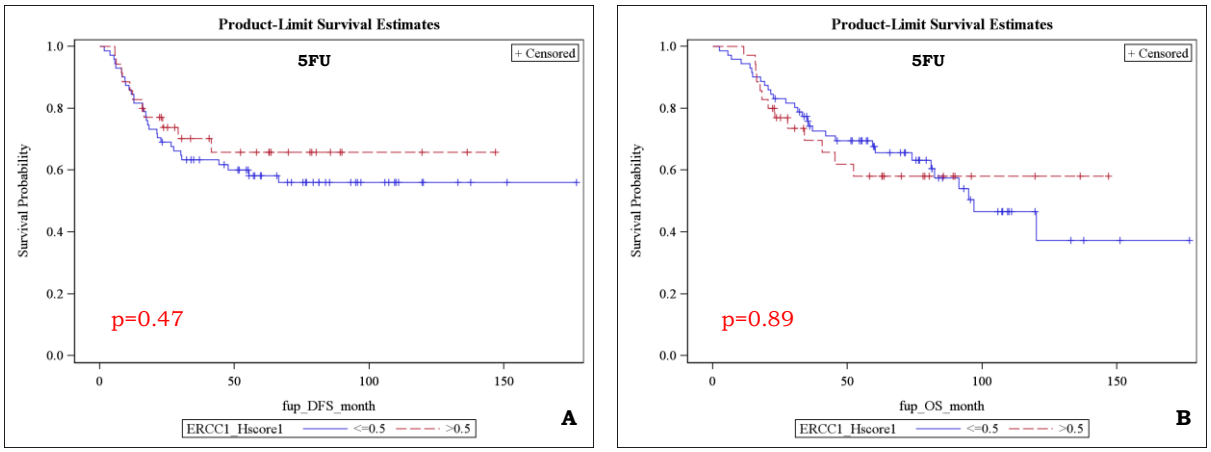

Figure 26: DFS (A) and OS (B) for ERCC1 negative (≤0.5) and positive (>0.5) expression detected by immunohistochemistry in 5FU treated patients.

Same absence of statistical association between ERCC1 immunohistochemical results and clinical outcome was observed in FOLFOX treated patients. The survival probability did not differ between ERCC1 positive (>0.5) and negative (≤0.5) patients, in both DFS (p=0.44) *(Figure 27A)* and OS (p=0.36) *(Figure 27B)*.

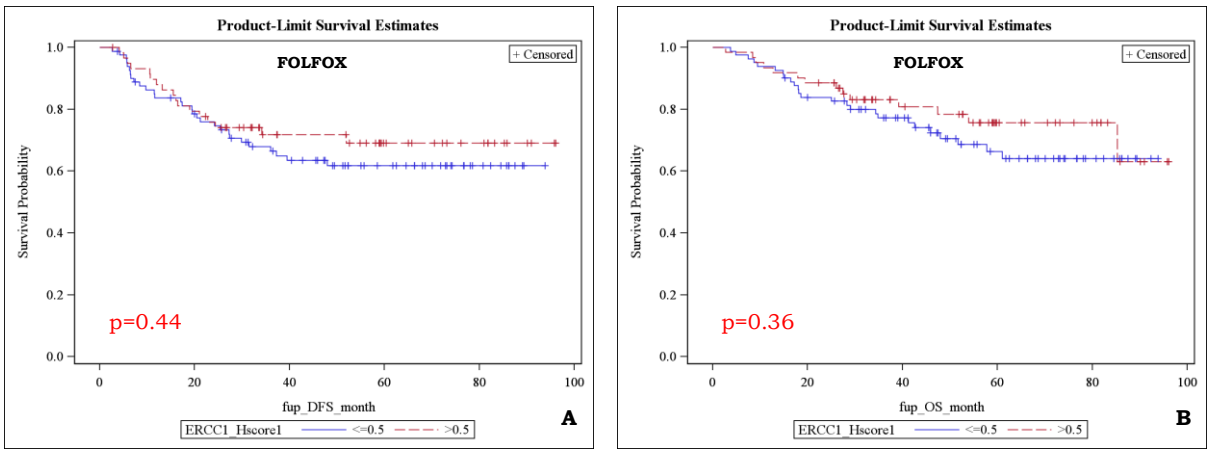

Figure 27: DFS (A) and OS (B) for ERCC1 negative (≤0.5) and positive (>0.5) expression detected by immunohistochemistry in FOLFOX treated patients.

Considering patients showing low levels (≤0.5) of ERCC1 protein and correlating them with the clinical outcome, we found no statistical difference between 5FU and FOLFOX treated patients for both DFS (p=0.58) *(Figure 28A)* and OS (p=0.79) *(Figure 28B)*.

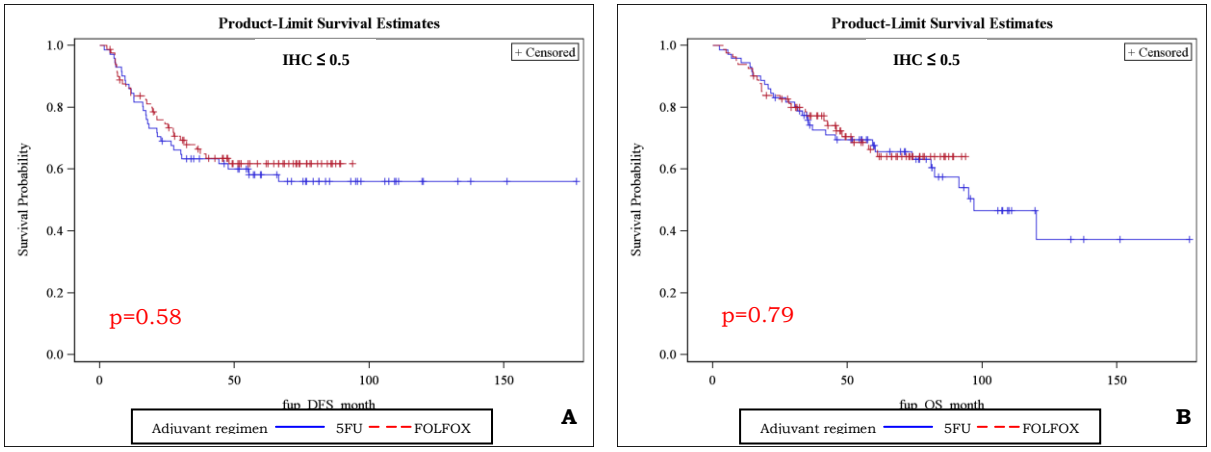

Figure 28: DFS (A) and OS (B) for cases showing low (≤0.5) ERCC1 protein expression level, stratified for the received treatment. 5FU= 5-fluorouracil; FOLFOX= 5-fluorouracil plus oxaliplatin.

Also observing clinical outcome for patients showing ERCC1 protein high levels (>0.5) no statistical difference was detected between 5FU and FOLFOX treatment, for both DFS (p=0.76) *(Figure 29A)* and OS (p=0.17) *(Figure 29B)*.

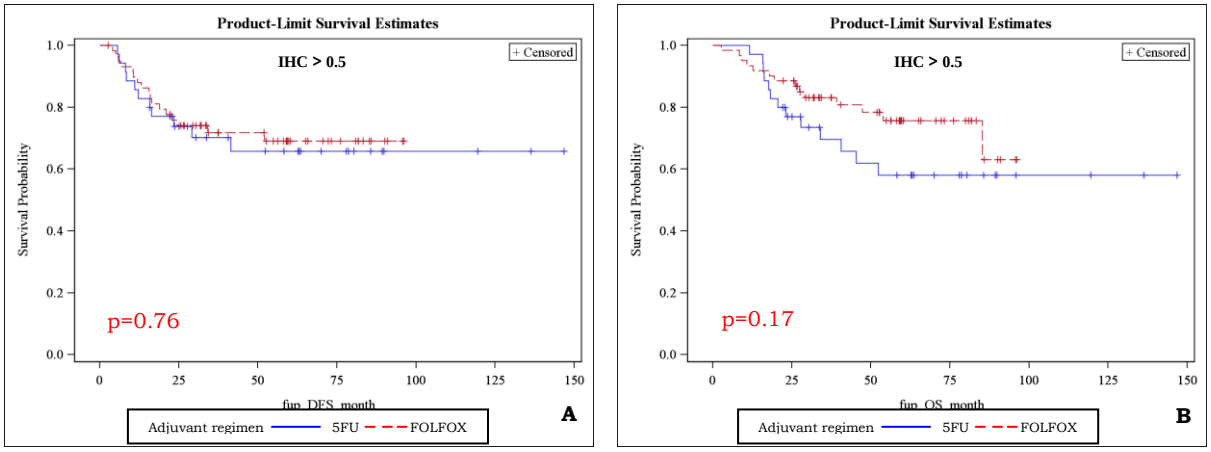

Figure 29: DFS (A) and OS (B) for cases showing high (>0.5) ERCC1 protein expression level, stratified for the received treatment. 5FU= 5-fluorouracil; FOLFOX= 5-fluorouracil plus oxaliplatin.

Similar results were obtained by using the 2 other ERCC1 scores.

Looking at the results obtained with the real-time PCR and considering the median value (1.20) as the cut-off point, we identified 42 (45%) patients in the 5FU group and 63 (55%) in the FOLFOX group showing a down-regulation (≤1.20) of ERCC1 mRNA levels.

The statistical analysis showed no difference in DFS (p=0.91) *(Figure 30A)* and OS (p=0.49) *(Figure 30B)* for 5FU treated patients with low (≤1.20) or high (>1.20) ERCC1 mRNA levels.

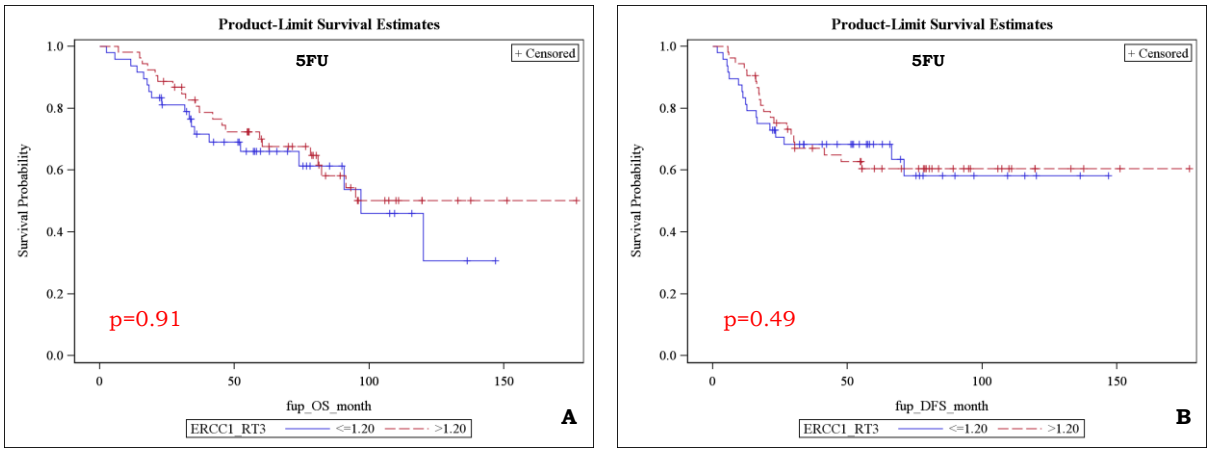

Figure 30: DFS (A) and OS (B) for ERCC1 negative (≤1.20) and positive (>1.20) mRNA expression levels detected by real-time PCR in 5FU treated patients.

The statistical analysis in FOLFOX treated patients showed a trend toward a better DFS (p=0.17) *(Figure 31A)* in patients characterized by low (≤1.20) ERCC1 mRNA levels. On the other hand, no statistical difference in OS (p=0.36) *(Figure 31B)* was observed for patients showing low or high ERCC1 mRNA levels.

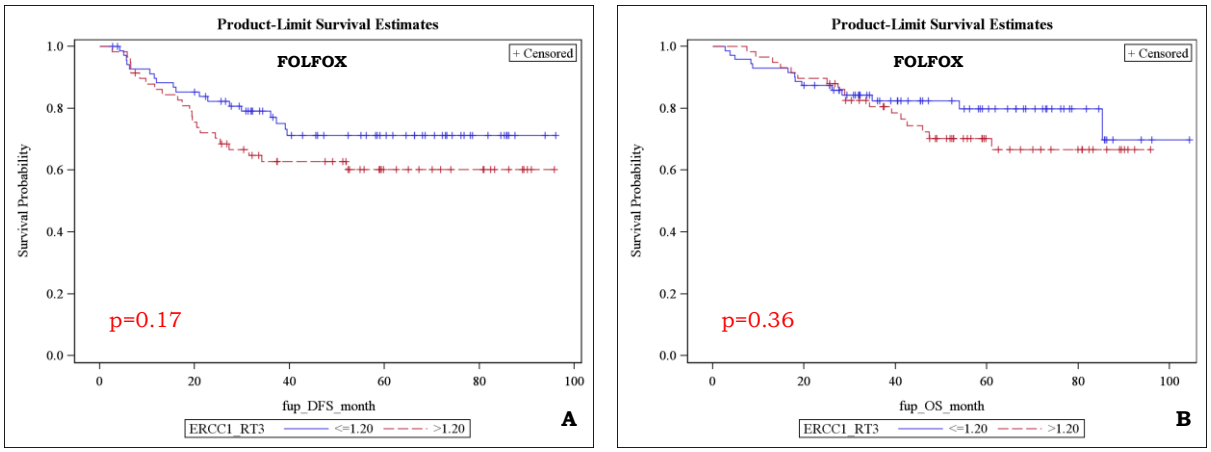

Figure 31: DFS (A) and OS (B) for ERCC1 negative (≤1.20) and positive (>1.20) mRNA expression levels detected by real-time PCR in FOLFOX treated patients.

Considering patients showing low ERCC1 mRNA levels (≤1.20) and correlating them with the clinical outcome, we found no statistical difference between 5FU and FOLFOX treated patients, even if we could observed a trend toward a better outcome in favour of FOLFOX treated patients, for both DFS (p=0.28) *(Figure 32A)* and OS (p=0.13) *(Figure 32B)*

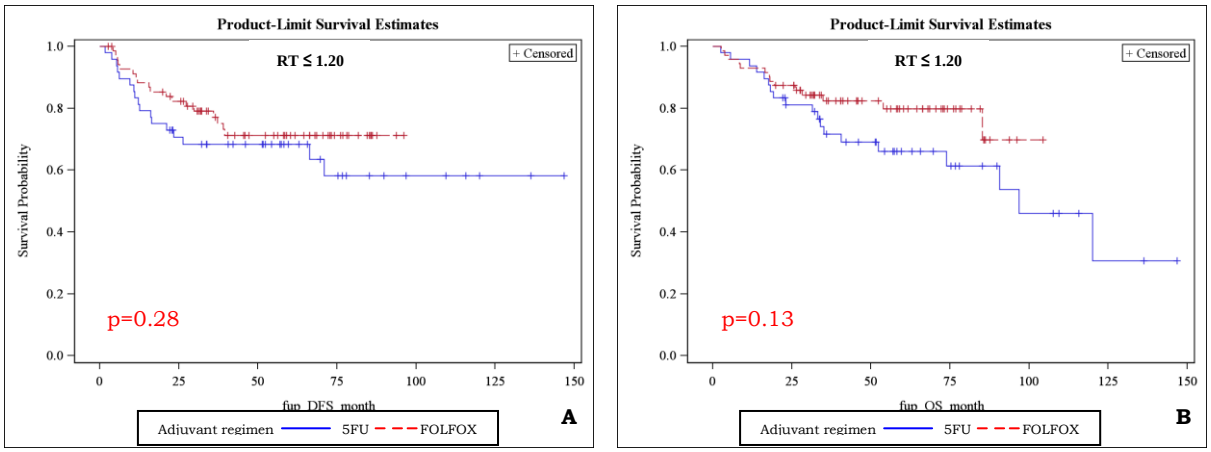

Figure 32: DFS (A) and OS (B) for cases showing low (≤1.20) ERCC1 mRNA levels, stratified for the received treatment. 5FU= 5-fluorouracil; FOLFOX= 5-fluorouracil plus oxaliplatin.

Observing clinical outcome for patients showing high (>1.20) ERCC1 protein mRNA levels no statistical difference was detected between 5FU and FOLFOX treatment, for both DFS (p=0.83) *(Figure 33A)* and OS (p=0.81) *(Figure 33B)*.

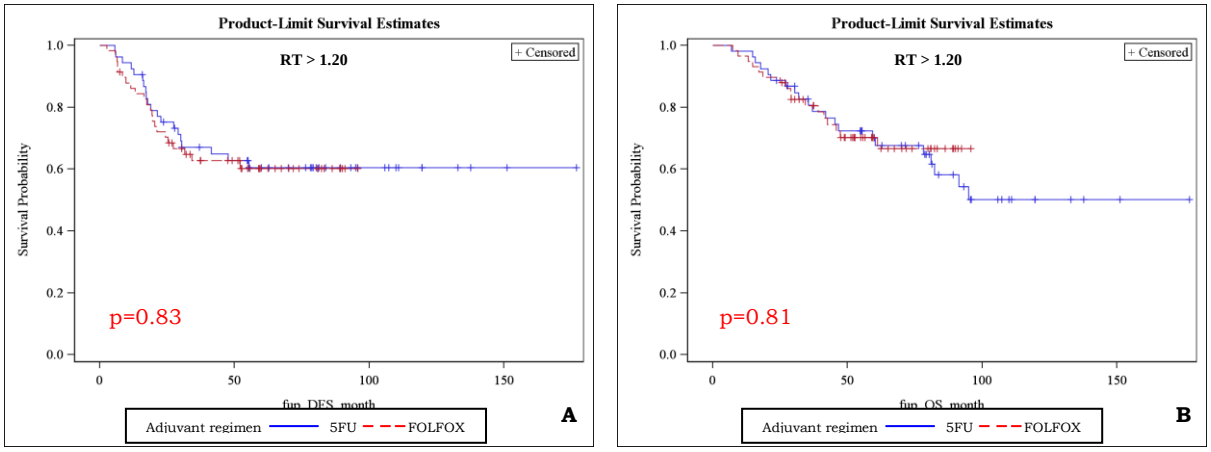

Figure 33: DFS (A) and OS (B) for cases showing high (>1.20) ERCC1 mRNA levels, stratified for the received treatment. 5FU= 5-fluorouracil; FOLFOX= 5-fluorouracil plus oxaliplatin.

4.5.5 Other correlations

The correlations between *ERCC1* polymorphism at codon 118 and *TP53* mutational status with clinical data are under evaluation.

Discussion

Nowadays the possibility to select patients that could benefit from a specific therapy is becoming essential, in particular in oncology where often we assisted to the onset of treatment resistance events and adverse side effects. Main efforts are aimed in the research of new potential prognostic and predictive markers, in particular for those diseases that are classified as the big killers, in order to better address patients to the proper chemotherapeutic treatment.

Colon cancer is the second cause of cancer-related death, accounting for over one million new cases per year worldwide (*Parkin DM et al, 2005*). In most patients it is diagnosed when still confined to the bowel and in these cases surgery resection is the main therapeutic option. In cases of advanced colon cancer, with a lymph node involvement, adjuvant chemotherapy is mandatory *(Wolmark N et al, 1988)*. The current standard of care for advanced (stage III) colon cancer patients is surgical resection, followed by 6-months 5FU-based chemotherapy or, more frequently, by oxaliplatin-based chemotherapy *(Andrè T et al, 2004)*. However, platinum-based treatments are associated with adverse side effects and, therefore, it is of high importance to find molecular markers able to identify patients who cannot benefit from this therapy, in order to avoid inefficacious treatments.

On this purpose we planned to analyze 230 patients from Switzerland and Argentina, characterized by a stage III colon cancer, who underwent adjuvant chemotherapy with 5FU alone, or in combination with oxaliplatin (FOLFOX), in order to establish the potential effect of some molecular markers on the two received treatments.

Patients characteristics of our cohort were in line with those reported in literature: the median age at diagnosis was 62 and the number of males and females was approximately similar. Furthermore, our cohort was equally subdivided in the two groups: 106 patients received 5FU as single agent and 124 were treated with FOLFOX regimen. This balanced distribution between the two chemotherapeutic options allowed us to make reliable considerations on the basis of statistical correlation with the clinical outcome in both groups of patients. The percentage of relapse was about 50% in both groups of patients and the median follow up was 50 months in the whole cohort.

As already reported in other studies, where an advantage in survival was observed for FOLFOX treatment when compared to 5FU regimen, we noticed a better DFS (51.3 months) and OS (55.4 months) for FOLFOX treated patients if compared to DFS (41.6 months) and OS (49.3 months) in the 5FU group *(Andrè T et al, 2004; Kuebler JP et al, 2007)*. However, the values are not statistically different, even if a slight trend is observed for OS (p=0.22), where the two curves corresponding to the two treatments appear well separated.

Overall, we must underline that the procedures with whom our cases had been processed were different between Argentinean and Swiss cases. The tumors of first group were fixed in Bouin while those of the second one in formalin. During molecular tests, we observed a high number of failures, if we compare our results with the median of the literature, in particular in the cohort recruited in Argentina.

Starting with molecular analyses, MSI is a predictor of good prognosis in a non-selected population of colon cancer including all stages. In our cohort, we observed that patients characterized by MSI (12%) showed a better DFS if compared to MSS cases. The value did not reach a statistical significance (p=0.17), in our opinion only due to the low number of analyzed cases. Therefore, we suggest that also in advanced stage III colon cancers, the evaluation of MSI status may be useful for the identification of patients with better prognosis.

On the contrary, the role of MSI in the prediction of efficacy of chemotherapy is still debated. In fact, it has been observed that in stage III colon cancer, patients who benefited the most from 5FU treatment showed a MSS pattern, with an increase of 5 months the DFS with respect to MSI patients *(Ribic CM et al, 2003)*. On the contrary, in another study no correlation between MSI and 5FU treatment was observed, for both DFS and OS *(Kim GP et al, 2007)*. Regarding the potential association of MSI with FOLFOX regimen, controversial data exist: two studies observed a better clinical outcome in MSI patients if compared to MSS cases, whereas two other works reported no differences in the clinical outcome for MSI and MSS patients *(Zaanan A et al, 2010; Zaanan A et al, 2011; Li P et al, 2013; Sinicrope AF et al, 2013)*.

In our cohort, we observed the trend for a better DFS in MSI patients treated with 5FU if compared to MSS patients (p=0.16). On the contrary, there was no difference in the clinical outcome for MSI and MSS patients in the FOLFOX group (p=0.67 and

p=0.86, DFS and OS respectively). Therefore our results are in contrast with those reported in literature by Ribic and Kim regarding the role played by MSI in 5FU regimen but we confirmed the absence of a relevant role of MSI in FOLFOX cases, as reported by Li and Sinicrope *(Ribic CM et al, 2003; Kim GP et al, 2005; Li P et al, 2013; Sinicrope AF et al, 2013)*. In conclusion, on the basis of all the assumptions reported to date, the role that MSI could play in the prediction of 5FU and FOLFOX treatments remains controversial.

A marker strictly associated with MSI is BRAF. In fact, it has been widely demonstrated that *BRAF* mutations, occurring in about 10% of colon cancer cases, are more frequently associated with MSI *(Wang L et al, 2003; Sinicrope AF et al, 2013)*. Our results, although obtained in a selected cohort of cases (i.e. only those with lymph nodes involvement), confirmed the data of the literature of both percentage of alteration and association with MSI (p=0.005). On the other hand, it has reported that mutations in *BRAF* gene usually confer a worse prognosis to colorectal cancer patients *(Fariña-Sarasqueta A et al, 2010; Ogino S et al, 2011; Yokota T et al, 2011)*. About this point, in our cohort we did not find any statistical association, probably due to the extremely low number of mutated cases or to the fact that our patients' series includes only advanced cases.

As far as the correlation with treatments efficacy is concerned, in a recent study it has been observed that *BRAF* mutations were associated with a shorter survival after recurrence in FOLFOX-treated patients when compared to *BRAF* wild-type cases *(Gavin PG et al, 2012)*. In our cohort, we did not observe any statistical difference in terms of DFS and OS between *BRAF* mutated and wild-type patients in both 5FU and FOLFOX groups. Therefore, the investigation of additional (and larger) cohorts is required to shed light on the role played by *BRAF* mutations in FOLFOX-treated patients.

We then focused our attention on another molecular marker, KRAS, on the basis of recent reports about the potential predictive role of *KRAS* mutations in oxaliplatin-based treated patients *(Bokemeyer C et al, 2009; Douillard JY et al, 2010)*.

The rate of *KRAS* mutations in our cohort was 28%, and the vast majority were identified in codon 12 (52/65). Percentage of alterations and types of mutations are in line with those reported in literature *(Edkins S et al, 2006)*.

When we matched the *KRAS* mutational status with clinical data of the whole cohort represented by advanced colon cancer patients, we observed that mutations in *KRAS* gene conferred a worse prognosis with respect to *KRAS* wild-type cases. The correlation with clinical outcome revealed in fact a trend towards a worse DFS (p=0.07) and a statistical worse OS (p=0.004) in *KRAS* mutated patients.

Then, we looked at the role of *KRAS* with respect to chemotherapies. The number of *KRAS* mutations identified in our cohort was balanced in 5FU and FOLFOX groups: 31 and 34 alterations, respectively. In 5FU-treated patients, we noticed a significantly worse DFS (p=0.04) and a trend towards a worse OS (p=0.07) for *KRAS* mutated patients if compared to those with a *KRAS* wild-type tumor. On the contrary, in FOLFOX group no difference was found on the basis of *KRAS* mutational status for both DFS (p=0.62) and OS (p=0.77). Furthermore, by stratifying our population on the basis of *KRAS* gene status, no difference was observed in wild-type patients treated with 5FU if compared to those treated with FOLFOX. On the contrary, in *KRAS* mutated patients a trend towards a worse DFS (p=0.28) and OS (p=0.20) was detected in 5FU group when compared to FOLFOX-treated patients. Our results suggest that curatively resected stage III colon cancer patients exhibiting wild-type *KRAS* status might benefit from 5FU alone. On the contrary, an oxaliplatin-containing regimen should be recommended in *KRAS* mutated patients.

Our results are supported by those observed in a preclinical study, where the percentage of apoptotic cells, under oxaliplatin treatment, transfected with a *KRAS* mutated vector, was significantly higher if compared to *KRAS* wild-type cells, thus suggesting that *KRAS* mutations may represent a predictor of oxaliplatin sensitivity in colon cancer *(Lin YL et al, 2012)*. A similar correlation was also observed in recent randomized trials (OPUS and PRIME) on metastatic colorectal cancer *(Bokemeyer C et al, 2009; Douillard JY et al, 2010)*.

The reason of the role of *KRAS* on the efficacy of oxaliplatin in colon cancer seems to be due to a link with ERCC1. In fact, at preclinical level, it has been shown that *KRAS* mutations are associated with ERCC1 down-regulation *(Lin YL et al, 2012)*. The possible correlation between *KRAS* and ERCC1 is not known yet. Those Authors proposed two possible mechanisms: 1) *KRAS* mutations may be responsible of an epigenetic mechanism leading to *ERCC1* promoter hypermethilation, causing a decrease of

ERCC1 expression; 2) there is a hypothetical unknown-ERCC1-activating factor that is inhibited by *KRAS* mutations, resulting in ERCC1 down-regulation. Therefore, we investigated ERCC1 expression at both protein (by IHC) and mRNA (by real-time PCR) level in our cohort of advanced colon cancer. In our study we did not confirm any correlation between *KRAS* mutation and ERCC1 expression, both at protein (also using different evaluation criteria) and mRNA level. The reason for this discordance is probably due to the fact that there are no guidelines helping in ERCC1 evaluation for both IHC and mRNA analysis, making ERCC1 assessment equivocal. In IHC analysis we used in fact scores on the basis of previous studies on lung cancer, and in gastric cancer, made by our group *(Olaussen KA et al, 2006; De Dosso S et al, 2013)*. Alternatively, we can think that the correlation between *KRAS* mutations and ERCC1 is valid only in specific cellular models or, at least, not in patients with a stage III colon cancer.

In general, it has been proposed that ERCC1 can play a predictive role for the identification of patients who may benefit from adjuvant chemotherapies and therefore it is useful to investigate this marker *per se* and not only in relation to *KRAS* mutational status. We identified positive ERCC1 protein expression levels in 40% of cases detected by IHC and in 49% of cases we identified ERCC1 positive mRNA levels, and our data are in line with those reported in literature *(Li P et al, 2013)*.

By correlating these molecular data with the clinical outcome in the whole cohort, we observed no statistical differences in DFS and OS when patients showed high or low-ERCC1 levels, in both protein and mRNA evaluation.

In our cohort we identified no difference in the clinical outcome for patients exhibiting ERCC1 high or low protein expression levels detected by IHC in both 5FU and FOLFOX groups. On the contrary, we detected a trend toward a better DFS (p=0.17) and OS (p=0.36) in FOLFOX treated patients when ERCC1 mRNA levels were negative. Our results are confirmed by the lonely work published on ERCC1 in FOLFOX-treated patients (including a South-Eastern Asian cohort) *(Li P et al, 2013)*. In this study, in fact, ERCC1 levels resulted to be highly predictive of which patients will benefit from the addition of oxaliplatin to 5FU for stage III colon cancer, in particular ERCC1 down-regulation corresponded to a better DFS (p=0.009) and OS (p=0.02) in oxaliplatin-based treated patients *(Li P et al, 2013)*. This is simply explained

by the fact that ERCC1 repairs oxaliplatin-induced adducts, so low ERCC1 levels result in a greater and more efficent oxaliplatin action. The association between ERCC1 expression and platinum-based chemotherapy response was also observed in lung cancer and in gastric cancer *(Chen W and Bepler G, 2013; De Dosso S et al, 2013)*. Therefore we could propose also ERCC1 expression analysis as a new marker for a better selection of patients to be addressed to oxaliplatin-based chemotherapy; but, of course, analyses of larger cohorts must be performed.

In addition to protein and mRNA levels, it has been suggested that a polymorphism occurring at codon 118 (AAT/AAC) of the *ERCC1* gene, may correlate with the ability of ERCC1 to repair the adducts induced by platinum-based compounds and its role as a marker of a better outcome in patients with colon cancer treated with oxaliplatin-based schemes was supported in several studies *(Stoehlmacher J et al, 2004; Viguier J et al, 2005; Ruzzo et al, 2007)*. However, the results are controversial: in a study it has been reported that TT genotype seemed to be associated with the most favourable survival, while the other two above-mentioned studies observed a significant correlation between CC genotype and better survival *(Stoehlmacher J et al, 2004; Viguier J et al, 2005; Ruzzo et al, 2007)*. In our cohort, the TT genotype was detected in 31% of cases, the CT genotype in 48% of patients and the CC variant in 21% of the whole cohort, and these percentage are in line with those reported *(Viguier J et al, 2005)*. The correlation between *ERCC1* genotype and clinical outcome in our cohort is now under evaluation.

Moreover, we also investigated the mutational status of *TP53*, because in the literature it has been proposed an increased risk of death and a worse prognosis ($p<0.001$) in those patients exhibiting *TP53* mutations *(Chen J et al, 2013; Sarasqueta AF et al, 2013)*, data not confirmed by another study where the presence of *TP53* mutations in colon cancer patients, was not a predictive factor of OS ($p>0.05$) *(Elsaleh H et al, 2000)*. Furthermore, it has been reported that stage III colon cancer patients who underwent adjuvant therapy with 5FU showed an improvement of 7-years survival when p53 was not over-expressed in respect to those patients exhibiting high p53 levels *(Ahnen DJ, 1998)*. On the contrary, in a preclinical study on cell lines, no relation between *TP53* status and 5FU sensitivity was observed ($p>0.05$) *(Brody JR et al, 2009)*. Therefore, the relationship between *TP53* status and the clinical outcome in colon cancer patients

remains controversial and deserves to be deeply investigated. In our cohort, we identified 44% of cases carrying a mutation in *TP53* gene; in particular we found the vast majority of alterations in exons 5 and 7, where the most common hotspots take place. The most frequent identified mutations were the R175H (in exon 5), the G245S, R248Q, R248W (in exon 7) and the R306X (in exon 8). Percentage of alterations and types of mutations are in line with data on colorectal cancer. The correlation with the clinical outcome is now under evaluation.

Finally, another marker, TS, was associated with the ability to identify patients who better benefit from 5FU administration. In fact, several studies demonstrated a better DFS and OS in patients characterized by low TS levels with respect to cases showing high percentage of TS *(Kornmann M et al, 2002; Aguiar S Jr, 2005)*. We therefore planned to set up the investigation of this marker. However, we found several problems during methodologies development (e.g. no complete reproducibility of the analysis of the same specimens with different experiments), and the time we have spent to solve these problems was much longer than expected. The analyses are currently ongoing.

In conclusion, looking at the whole cohort, we can confirm a better clinical outcome for adjuvant colon cancer patients treated with FOLFOX regimen with respect to 5FU treatment. MSI could be a useful tool indicating a better prognosis also for advanced colon cancer but its role in predicting 5FU or FOLFOX efficacy remains controversial. In addition, we propose to assess ERCC1 mRNA expression analysis before the administration of oxaliplatin-based chemotherapy, in order to early identify the patients who may benefit the most from this treatment.

Finally, we suggest that *KRAS* mutational status could help clinicians in selecting the best chemotherapeutic treatment in the adjuvant setting: only *KRAS* mutant patients should be treated with a platinum-based chemotherapy, while patients whose tumour is *KRAS* wild-type could be treated with 5FU alone, thus preventing adverse side effect in a consistent number of cases. Our results, of course, deserve confirmations.

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