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Topoisomerase I and II Expression in Recurrent Colorectal Cancer Cells: A Dubious Matter

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1. Introduction

DNA topoisomerases are enzymes which alter and modify the topology of double-stranded DNA, without changing the sequence of the structural units of DNA, namely the nucleotides. They act by transiently breaking and then religating the DNA helix. Therefore, they unwind the double helix, relaxing the supercoiled DNA, and they allow DNA strands or double helices to pass through each other. Topoisomerases are essential for replication, transcription, translation and recombination of DNA because, relaxing the double helix, they facilitate the function of other enzymes, like DNA and RNA polymerases. Topoisomerases are the magicians of the DNA world, as they practically solve all the topological problems occurring during every aspect of DNA metabolism (Gupta et al., 1995; Pommier et al., 1998; Stewart et al, 1998; Champoux et al., 2001; Burden et al., 1998; Kellner et al., 2003; Wang et al., 2002).

The first topoisomerase discovered, in 1971, was *E. coli* topoisomerase I, originally known as ω protein (Wang, 1971). Next year there was the discovery of eukaryotic topoisomerase I in nuclei extract from secondary mouse-embryo cells (Champoux et al., 1972) In 1976, DNA gyrase (topoisomerse II) was purified from *Escherichia col*i cells (Gellert et al., 1976a). Topoisomerases have been categorized in two basic families, according to their structure and mechanism of action: type I topoisomerases and type II topoisomerases. Type I topoisomerases mediate transient breaks in one of the DNA strands. They are further classified in the subfamilies IA and IB. Type IA topoisomerases form a covalent intermediate with the 5'-phosphoryl end of DNA, while type IB topoisomerases form a covalent intermediate with the 3'-phosphoryl end of DNA (Champoux et al., 2001).

On the other hand, type II topoisomersases transiently cleave both of the two DNA strands. They are also further classified in the subfamilies IIA and IIB on the basis of differences in their protein structure. In human and higher eukaryotic organisms, three groups of topoisomerases have been described. The first group includes topoisomerase I and mitochondrial DNA topoisomerase, which are type IB enzymes. The second group includes topoisomerases IIα and IIβ, which are type II enzymes. The third group, which was later discovered, includes topoisomerases IIIα and IIIβ, which are type IA enzymes (Lodge et al., 2000; Kwan et al., 2001; Hanai et al., 1996). Topoisomerase IV has also been described, a bacterial type II topoisomerase (Kato et al., 1990). More recently, topoisomerase V has been described, which is a prokaryotic counterpart to the eukaryotic topoisomerase I (Slesarev et al., 1994).

Topoisomerase I is the well known Scl-70 antigen, against which high autoantibodies titters are developed in diffuse cutaneous systemic scleroderma (Shero et al., 1986; Guldner et al., 1986; Shero et al., 1987). Antibodies against topoisomerase IIα are detected in localized scleroderma (Takehara et al., 2005). Topoisomerases IIIα and IIIβ are possibly involved in the pathogenesis of Bloom, Werner and Rothmund-Thomson syndromes, which are associated with genetic instability (Kwan et al., 2001; Raynard et al., 2006).

Similar enzymes, as mentioned above, do exist in the prokaryotic cell (bacterial topoisomerases-gyrases) (Wang, 1971; Gellert et al., 1976a; Brown et al., 1979), and in viruses (e.g Vaccinia virus topoisomerase I) (Shaffer et al, 1987; Shuman, 1998). The clinical significance of bacterial gyrases is great, as they constitute targets for antibiotic drugs like novobiocin, nalidixic acid and new quinolones (Gellert et al., 1976b; Sugino et al., 1977; Smith, 1986; Maxwell, 1992).

Topoisomerase IIα and topoisomerase I are the best studied human topoisomerases, and constitute molecular targets for well known and widely used antineoplastic drugs (Pommier et al., 1998; Burden et al., 1998).

2. Topoisomerase I

Human DNA topoisomerase I is essential for vital cellular processes, namely DNA replication, transcription, translation, recombination and repair. It is a 91 kDa monomeric polypeptide which consists of 765 amino acids. It is encoded by a single copy gene which is located on chromosome 20q12-13.2 (Champoux et al., 2001). It catalyzes the relaxation of both positively and negatively supercoiled DNA, while bacterial topoisomerase I catalyzes the relaxation of negatively supercoiled DNA. Superhelix density for most natural DNA molecules ranges from -0.03 to -0.09 (Bauer et al., 1980). The negative sign means that DNA superhelices are left-handed. In other words, superhelices are derived from reverse winding of the helix. During the process of transcription, RNA polymerase follows the helical path along DNA double strand, having the tendency to generate positive supercoils in front of it and negative supercoils behind it. The accumulation of such supercoils could block the process of transcription, harming cell viability. Therefore, toposomerase I acts as a swivel to relieve the torsional strain caused by the generation of positive supercoils upstream and negative supercoils downstream of the moving RNA polymerase. It gives the solution, removing any undesirable supercoil (Liu et al., 1987; Wang et al., 1993).

During the process of replication, topoisomerase I has a similar function. There is a tendency of positive supercoils accumulation. Topoisomerase I relaxes the supercoils, relieving the torsional strain of the DNA molecule (Avemann et al., 1998; Hsiang et al., 1989). Topoisomerase I has also a role in the process of DNA recombination, DNA repair, and mitotic chromosome condensation (Pommier et al., 1998; Bullock et al., 1985; Wang et al., 1991; Shuman et al., 1989; Castano et al., 1996; Subramanian et al., 1998).

Topoisomerase I does not require ATP for its action, in contrast to topoisomerase II, which does need ATP (Wang et al., 1969). In the active centre of the enzyme there is the active site tyrosine (Tyr-723) of which the hydroxyl group performs a nucleophilic attack on the phosphodiesteric bond of one of the two DNA strands. The phosphodiesteric bond is broken now. The enzyme remains covalently attached to the 3' end of the broken strand, creating a transient covalent complex, known as cleavable complex. The 5' hydroxyl group of the broken strand remains free. The DNA molecule can now rotate around the intact strand. This rotation leads to the relaxation of the abovementioned positive and negative supercoils.

The whole process is completed as the exposed 5′ hydroxyl group performs a nucleophilic attack on the transient phosphotyrosine linkage. The active site tyrosin is detached from the 3′ end. The cleaved strand is resealed (Pommier et al., 1998; Sari et al., 2005).

In summary, topoisomrasre I unwinds and uncoils the DNA supercoiled double helix by transiently cleaving one of the two strands and allowing rotation over the other. In the end it reseals the cleaved strand.

Topoisomerase I is not essential for viability in yeast (Thrash et al., 1984; Uemura et al., 1984). On the contrary, it is essential for embryonic development in *Drosophila melanogaster* and mouse (Lee et al., 1993; Morham et al., 1996).

By contrast to topoisomerase II, topoisomerase I levels are not cycle-specific and remain relatively constant throughout the cell cycle (Heck et al., 1988). Topoisomerase I seems to be expressed in all cells, even those which do not divide. However, the enzyme levels are higher in cancer tissues, compared to the adjacent normal tissues. This fact renders topoisomerase I an attractive target for chemotherapy drugs (Bronstein et al., 1996; Husain et al., 1994).

Topoisomerase I catalytic activity was the first parameter of topoisomerase I evaluable in biomedical studies and it was detected in all normal tissues at a fairly constant level. Two more parameters were added in the investigational process: topoisomerase I immunoprotein levels, estimated by Western Blotting analysis, and topoisomerase I gene expression (toposomerase I mRNA), evaluated by Northern Blotting analysis. There seems to be a good correlation between topoisomerase expression and the catalytic activity of the enzyme (Bronstein et al., 1996; Husain et al., 1994). Using the abovementioned parameters and methods, elevated topoisoomerase I levels were detected in ovarian cancer, cervical cancer, colorectal cancer, prostate cancer, malignant melanoma, lymphoma (Bronstein et al., 1996; Husain et al., 1994; Van der Zee et al., 1991; Van der Zee et al., 1994; Perego et al., 1994; McLeod et al., 1994; Goldwasser et al., 1995; Guichard et al., 1999; Giovanella et al., 1989; Florell et al., 1996). On the other hand, there was no elevation in topoisomerase I levels detected in lung cancer, breast cancer, renal cancer, and rhabdomyosarcoma (Bronstein et al., 1996; Husain et al., 1994; McLeod et al., 1994). With regard to colorectal tumors, topoisomerase I levels have been found to demonstrate 5-35-fold increases in the cancer tissue, compared to the adjacent normal colonic mucosa (Bronstein et al., 1996; Husain et al., 1994). In 1997, a new evaluable parameter of topo I appeared in biomedical studies: topo I expression, evaluated by immunohistochemistry in paraffin embedded human tissues. Since then, topo I expression has been estimated in several neoplastic tissues. In fact, elevation of topo I was found in ovarian carcinomas (43% of tumors examined); colorectal carcinomas (ranging from 43% to 86% in different studies) ; testicular tumors (30-38% of seminomas, 30% of embryonal carcinomas, but 100% of teratomas and yolk sac tumours); urinary bladder carcinomas (77%); renal cell carcinomas (ranging from 36-100% according to histological grade); malignant melanomas (41,6%); gastric carcinomas (68%); sarcomas (13%); breast carcinomas (41%); oral dysplasias (79%) and squamous cell carcinomas (92%) (Holden et al., 1997; Boonsong et al., 2002; Staley et al., 1999; Paradiso et al., 2004; Monnin et al., 1999; Coleman et al., 2001; Coleman et al., 2000; Berney et al., 2002; Lynch et al., 2001; Gupta et al., 2000; Lynch et al., 1998; Hafian et al., 2004; Coleman et al., 2002). As far as topoisomerase I immunoreactivity in normal tissues is concerned, this appeared to be strongest in the germinal centres of the tonsil and in the lymphocytes of colonic mucosa, while it was also detected in the glandular colonic epithelium (Holden et al., 1997).

3. Topoisomerase II

Topoisomerase II is a ubiquitous enzyme which is essential for the viability of all eukaryotic organisms and plays a crucial role in literally every aspect of DNA metabolism and chromosome organization. In contrast to topoisomerase I, which is actually monomeric, topoisomerase II exists in two homologous but different isoforms, designated IIα and IIβ, which are closely related. The two isoforms share homology in the amino acid sequence (up to 70%), but they are encoded by separate genes. Topoisomerse IIα is the isoform originally described and characterized in mammalian species. It has a molecular mass of 170 kDa and is encoded by a gene which is located on chromosome 17q21-22. Topoisomerse IIβ has a molecular mass of 180 kDa and is encoded by a gene which is located on chromosome 3q24. Both of the proteins exist as homodimers (Burden et al., 1998; Kellner et al., 2003; Pommier et al., 2001; Jenkins et al., 1992; Austin et al., 1993).

Topoisomerase IIβ concentrations are relatively constant during the cell cycle. On the contrary, topoisomerase IIα levels are tightly associated with the proliferative state of the cell, and increase 2-3 fold during G_2/M phase. This increase takes place in rapidly proliferating tissues, while quiescent populations demonstrate low enzyme levels (Heck et al., 1988). Therefore, it is believed that the β isoform is responsible for the "housekeeping" functions of the enzyme, while the α isoform seems to be the type II enzyme which unlinks daughter chromosomes following replication. A small percentage of the total topoisomerase II pool in mammalian cells exists as α/β heterodimers (Biersack et al., 1996).

The enzymological characteristics of all eukaryotic type II appear to be similar. Each topoisomerase II monomer can be divided in three domains. The N-terminal domain, which includes the first 660 amino acids of the enzyme, is homologous to the B subunit of DNA gyrase and contains sequences for ATP binding. The central domain of the enzyme, which extends to amino acid 1200, is homologous to the A subunit of DNA gyrase and contains the active site tyrosine that forms the covalent bond with DNA during scission. The C-terminal domain of the enzyme varies from species to species and doesn't seem to have homology with DNA gyrase. The physiological function of this domain remains unclear. It possibly has a role in modulating the DNA cleavage/ligation reaction of the enzyme (Wang et al., 1996; Berger et al., 1998; Dickey et al., 2005).

Topoisomerase II works in a way similar to topoisomerase I, with the difference that it cleaves both strands of the nucleic acid substrate, allowing the passage of an intact double helix through the break. The enzyme Topoisomerase II is able to catalyze the relaxation of both positively and negatively supercoiled DNA. Its catalytic action begins with the simultaneous cleavage of both of the two DNA strands. The active site tyrosins (one for each monomer of the homodimer) covalently bind to the 5' end of the broken strands and create the transient complex which is known as cleavable complex. Till this step, energy from ATP hydrolysis is not required. The protein dimer is stabilized with disulfide bridges that create a gate through which we have the passage of another intact double-stranded DNA helix. This passage takes place at the expense of ATP hydrolysis. The whole procedure is completed with the resealing of the cleaved double strand (Roca et al., 1994; Schultz et al., 1996; Lindsley et al., 1996).

As a result of this DNA passage mechanism, topoisomerase II is able not only to remove negative or positive DNA supercoils, but also to unlink intertwined pairs of newly replicated chromosomes. This extra feature of topoisomerase II is important for chromosome organization and segregation. Topoisomerase I doesn't share this feature (Roca et al., 1996).

As mentioned above, topoisomerase IIα levels are cell cycle-dependent, with maximum concentrations measured during the G2/M phase. That's why the percentage of positive of positive what? for topoisomerase IIα cells practically represents the percentage of dividing cells. Thus, several studies have displayed that topoisomerase IIα levels may act as a reliable marker of cell proliferation in tumors. Topoisomerase IIα has been used as a proliferation marker in several studies, including patients with colorectal cancer (Monnin et al., 1999; Holden et al., 1995; Nakopoulou et al., 2001; Gibbons et al., 1997).

Concerning normal tissues, topoisomerase IIα levels are higher in tissues with proliferating cells (eg. spermatocytes, germinal cells, and proliferative endometrium) references?. In contrast, no detectable topoismerase IIα was detected in terminally differentiated tissues, e.g cerebral cortex, skeletal muscle, and nerve (Bauman et al., 1997). Concerning neoplastic tissues, high levels of topoisomerase IIα are observed in biologically aggressive or rapidly proliferating tumors, like high-grade lymphomas or seminomas. Topoisomerase IIα is detectable in both the cell nucleus and cytoplasm. Topoisomerase IIβ is localized both in the nucleoli and the nucleoplasm. It is ubiquitously expressed in vivo and it is present in quiescent cell populations (Turley et al., 1997). Concerning normal colon mucosa, topoisomerase IIα is expressed only in the lower crypt zone. In adenomas, the topoisomerase IIα expression is expanded in the upper crypt region, while it is diffuse in carcinomas (Fogt et al., 1997).

Topoisomerase II constitutes the molecular target of a great number of antineoplastic drugs, which are widely used in cancer chemotherapy, including anthracyclins and epipodophyllotoxins. These drugs constitute substrates of the classic MDR proteins. It is interesting that topoisomerase II is implicated in a type of multiple drug resistance which is called atypical MDR. There is actually a type of multiple drug resistance only to anthracyclins and epipodophyllotoxins, not the vinca alkaloids and colchicine. This type has no relation with classical MDR (Borst et al, 1995). This atypical MDR is attributed to mutations associated with topoisomerase II. There are two basic types of such mutations:

1. Mutations which lead to low levels of the enzyme. Drugs targeting topoisomerase II stabilize the cleavable complexes between the enzyme and DNA, creating permanent breaks of the double strand. Reduced levels of topoisomerase II lead to reduced DNA strand breaks, therefore reduced drug activity (Zijlstra et al., 1990; Cole et al., 1991; Sullivan et al., 1987).

2. Mutations leading to a qualitatively modified topoisomerase II, less sensitive to the chemotherapy drugs (Beck et al., 1993; Glisson et al., 1987).

4. Topoisomerase I inhibitors

There are a lot of inhibiting topoisomerase I (Pommier et al., 1998; Wang et al., 1997). In clinical practice only camptothecin derivatives are used.

Camptothecin (CPT) is an alkaloid found in the wood and bark of the Chinese tree Camptotheca acuminata (Nyssaceae) (Wall et al., 1966). Wall and Perdue isolated CPT from Camptotheca acuminate during the '60s. CPT and its derivatives were also found in other plant families.

In the early phase-I and II clinical studies regarding CPT, the water soluble sodium salt NSC-100880 was used. Despite the antineoplastic activity, the studies were suspended due to unacceptable hematologic (myelosupression) and non hematologic (hemorrhagic cystitis) toxicity (Gottlieb et al., 1970; Muggia et al., 1972; Moertel et al., 1972; Gottlieb et al., 1972;

Schaeppi et al., 1974). Later, it was shown that the cytotoxic activity of camptothecin, that has pentacyclic structure, was attributed to its E-ring lactone. When the E-ring opens to form hydroxyacid, the drug becomes inactive. In water solutions the inactive open form is favored (Slichenmyer et al., 1993). On the other hand, the low water solubility of the drug was the main reason for its toxicity. Therefore, an effort began having to do with the synthesis of CPT derivatives that combine the maximum possible water solubility with the best cytotoxic activity. Semisynthetic water soluble derivatives were produced. The most important of them were topotecan (TPT) and irinotecan (CPT-11). These new drugs were developed with modification of the A-ring of the camptothecin molecule (Kerrigan et al., 2001).

Camptothecin derivatives act causing irreversible breaks on the DNA strands. They actually act stabilizing the cleavable complexes formed by topoisomerase I, which normally have short half life. This stabilization takes place bringing a guanine at the 5' end of the cleaved DNA. While the stabilization is irreversible, it causes irreversible break of the double strand, when the replication fork meets a cleavable complex (Covey et al., 1989; Hsiang et al., 1989; Pommier et al., 1996; Jaxel et al., 1991). When topoisomerase I levels are higher, the cleavable complexes are more frequent, and the DNA strand breaks are more frequent, too. These breaks lead to the cell cycle arrest at S/G_2 phase, activation of apoptotic mechanisms and cell death (Hsiang et al., 1988). That's why camptothecin derivatives are cytotoxic in presence of active DNA replication or RNA transcription. Cells synchronized in S phase are 1000-fold more sensitive than in phases G_1 and G_2/M. Despite the fact that topoisomerase levels remain stable during the cell cycle, camptothecin derivatives constitute cycle-specific drugs (Liu et al., 1983; D'Incalci et al., 1993).

The term "inhibitors" when we refer to topoisomerase I inhibitors is somewhat catachrestic. Camptothecin derivatives actually do not inhibit topoisomerase I but they use the enzyme function in order to transform the enzyme into a cell poison.

Irinotecan is bioactivated in liver by carboxylesterase to the active metabolite SN-38. SN-38 is 1000-fold more active (Slichenmyer et al., 1993). Both irinotecan and SN-38 are susceptible to pH – dependent reversible hydrolysis and transformation of the active closed ring (lactone) to the open form of hydroxyacid. Acidic pH favors the active form of closed ring. The open form not only lacks the ability of cleavable complexes stabilization, but also the ability of entrance into the cell via the cell membrane.

The active metabolite SN-38 is responsible not only for the antineoplastic activity of irinotecan, but also for the side-effects. Late diarrhea is the most important side effect and it often does not respond to common anti-diarrheic drugs. SN38 is further metabolized by the UDP-glucuronosyltransferase 1A1 (UGT1A1) enzyme to the inactive form SN38 glucuronide (SN38-Glu), which is excreted in the gastrointestinal lumen via bile. It is believed that intestinal bacteria produce β-glucuronidase, that hydrolizes SN38-Glu to the active form SN38. SN38 causes intestinal mucosa injury, that leads to late diarrhea. At the same time, the non-metabolized irinotecan constitutes a weak acetylcholinestarase inhibitor and may cause acute cholinergic symptoms, among which is the early diarrhea in some patients (Abigerges et al., 1994; Kehrer et al., 2001; Lokiec et al., 1995; Takasuna et al., 1996; Gupta et al., 1994).

Irinotecan received in 1996 and 1998 FDA approval for treatment of metastatic colorectal cancer after failure of treatment with fluorouracil. The importance of the drug was proved in two European randomized studies (Cunningham et al., 1998; Rougier et al., 1998). In 2000,

irinotecan received FDA approval for first line treatment in metastatic colorectal cancer, combined with fluorouracil/leucovorin (Saltz et al., 2000; Douillard et al., 2000).

Topotecan is the second camptothecin derivative. It is itself an active drug. A low percentage of the drug is metabolized by microsomal enzymes to N-demethyltopotecan, which is also an active metabolite. In contrast to irinotecan, which is metabolized in liver, topotecan is mainly excreted in urine. Topotecan received FDA approval as second line therapy in metastatic ovarian cancer (1996) and in SCLC (1998).

5. Topoisomerase II inhibitors

Topoisomerase II inhibitors are cytostatic drugs widely used in clinical practice since decades (Hande et al., 1998). According to their mechanism of action, they are divided in two broad categories. The first category includes DNA intercalators, which intercalate between the base pairs of DNA, disrupting DNA function. This category includes cytotoxic antibiotics like anthracyclines (eg. doxorubicin, epirubicin, mitoxantrone) and aminoacridines, among which amsacrine is the main drug (Bailly, 2000). The second category includes substances which do not act with an intercalation mechanism. Epipodophyllotoxins and some isoflavones, like genistein belong to this category. Genistein is included in soy, and it is possibly responsible for the low incidence of breast cancer, prostate cancer and colorectal cancer in Asian populations (Barnes et al., 1995; Stoll et al., 1997).

Quinolones, the well known family of broad spectrum antibiotic, are also topisomerase II inhibitors. They are the only group of drugs with activity against both eukaryotic topoisomerase IIα and its prokaryotic homologue, bacterial gyrase. Quinolones are not used as antineoplasic drugs, till now, but they include widely used antibiotics like ciprofloxacin and norfloxacin (Burden et al., 1998; Maxwell et al., 1992; Robinson et al., 1991; Robinson et al., 1992).

Epipodophyllotoxins were synthesized in an effort of amelioration of podophyllotoxin activity. Podophyllotoxin is a substance which constitutes extract of the plant *Podophyllum peltatum*, known by the American Indians for its emetical, cathartic, and anthelminthic activity (Mantle et al., 2000). Two semisynthetic glucosides were synthesized from podophyllotoxin: etoposide and tenoposide. Etoposide is produced with the attachment of podophyllotoxin to a glucopyranoside with a methyl group, while tenoposide is produced with the attachment of podophyllotoxin to a glucopyranoside with a thenyliden group. This simple modification causes an important change in the way of action of the drug. Therefore, while podophyllotoxin acts on microtubules that form mitotic spindle, etoposide and teniposide act as topoisomerase IIα inhibitors (Schilstra et al., 1989).

Etoposide and teniposide act on topoisomerase IIα, in a way similar to which irinotecan and topotecan act on topoisomerase I. They actually take advantage of the normal topoisomerase IIα action, in order to transform it into a cell poison. The whole process takes place during stabilization of DNA- topoisomerase IIα complexes, which induces double strand breaks (Burden et al., 1998). Cells in S and G_2 phases of the cell cycle are more sensitive to epipodophyllotoxins. However, in contrast to the camptothecins derivatives, which are characterized by high cycle specificity, DNA synthesis inhibition only partially affects epipodophyllotoxin induced cytotoxicity (Holm et al., 1989).

Etoposide is a widely used antineoplastic drug highly active in germ cell tumors, ovarian cancer, SCLC, NSCLC, non-Hodgkin lymphoma, acute leukemia, Ewing sarcoma, Kaposi sarcoma, neuroblastoma. Tenoposide is less used in clinical practice.

6. Original research

6.1. Pilot study
6.1.1 Materials and methods

6.1.1.1 Patients' characteristics – study and control groups

In the study we are describing in this chapter, a total of twenty-five patients were included. Those patients had colorectal cancer which had recurred following surgery and chemotherapy. Specifically, patients had undergone complete surgical resection of the primary tumour and subsequently were submitted to a 5-FU-based adjuvant chemotherapy regimen, postoperatively. Patients were followed-up until recurrence. When recurrences occurred, patients underwent a second surgical resection. Biopsy specimens from both surgical procedures for each patient were, therefore, collected, so that at the end of the study we evaluated two histological specimens from each patient: one from the primary tumour location (i.e. before the administration of 5-FU-based adjuvant chemotherapy) and a second one from the neoplastic tissue at the recurrence site (i.e. following the 5-FU-based chemotherapy regimen).

In order to be able to make comparisons we needed a control group, so we selected a group of twelve patients with colorectal cancer who underwent resection after initial diagnosis, but received no 5-FU-based adjuvant chemotherapy. Patients in the control group were age and gender mached and had also similar tumour characteristics with patients included in the main study group. For each patient in the control group two biopsy specimens were available for evaluation since they were submitted to surgery following recurrence.

6.1.1.2 Histologic evaluation – immunochemistry

For each biopsy specimen, the expression of topoismerase-I was quantified by means of standard three-step immunohistochemistry on paraffin embedded sections. For this purpose the Topogen Topo I Monoclonal Antibody (2012-3) was used; this is a monoclonal mouse antibody (IgG2b isotype). The epitope of the antibody has not been mapped. Normal human tonsil tissues served as positive control. Histological sections were examined by a single investigator with no previous knowledge of the clinical status of the examined specimen.

During histologic examination, immunostaining for topoisomerase I was graded according to the percentage of tumour cells with positive staining (- and ± for <5%, + for 5%-50% and ++ for 50% to 75%) and according to the intensity of staining (weakly positive, moderately positive, or strongly positive). Only specimens with strongly immunoreactive nuclei were considered as a positive biopsy for topoisomerase I; weak and moderate intensity of staining was considered as negative for the expression of topoisomerase I.

6.1.1.3 Statistical Analysis

Following this initial evaluation, the sections were examined in pairs (one section for the first surgery and one section for the second surgery for each patient) during the statistical analysis. For the latter, we used McNemar's paired chi-square test to assess the possible modification of the levels of topoisomerase I following chemotherapy with 5-FU. Fisher's exact test was performed in order to assess the possible relationship of the topoisomerase I increase with gender, Duke's stage, grade of differentiation and tumour localization. Mann-Whitney U-Test was performed in order to assess possible relationship between the age of patients and changes of topoisomerase I levels as well as to investigate possible correlations between the relapse free interval (RFI) and alteration of topoisomerase I levels.

6.1.2 Results

6.1.2.1 Topoisomerase I level increase

Patients were categorized into two groups according to the increase of topoisomerase I levels (Figure 1). Using McNemar's paired chi-square test, we found that malignant cells from the tumour recurrences were characterized by the presence of a statistically significant increase in the levels of topoisomerase I when compared to the cells from the primary tumours (2-sided p=0.01) (figure 1).

6.1.2.2 Correlation with tumour parameters and demographics

The raise in topoisomerase I levels observed in the previous section/Figure 1 did not demonstrate significant correlations with Duke's stage (Fisher's Exact Test p-value = 0.496), grade of differentiation (p value = 0.661), tumour localization (p value = 0.072), or patients' gender (p value = 0.434). On the other hand, a statistically significant relationship was observed between the age of patients and the increase in topoisomerase I levels (p = 0.011). Using Mann-Whitney U-Test, patients with an increase in topoisomerase I levels were found to be older in age (median=62.5 years) than patients without increase in topoisomerase I levels (median=50 years) (p = 0.038).

Moreover, patients with an increase in topoisomerase levels had a median relapse free interval (RFI) of 17.5 months, while patients without such increase had a median RFI of 16 months. This difference in the RFIs between the two groups was not statistically significant (p = 0.493).
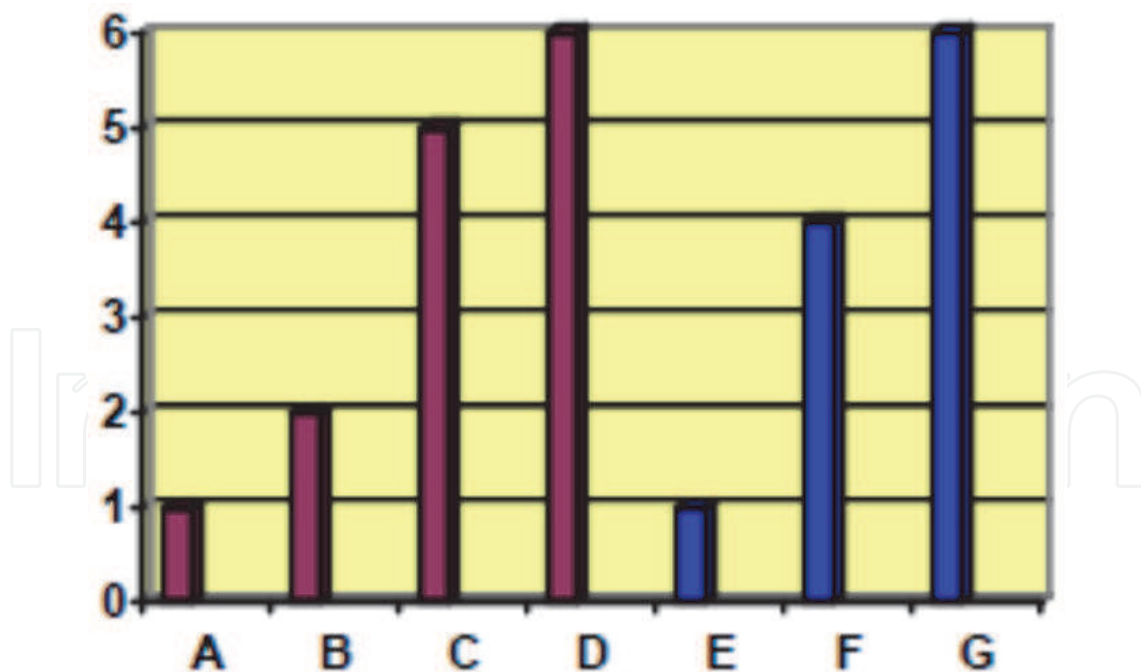
Y: 0-6 = Number of patients (n=25)
X: A (□ ►++), B (+►++),   C (-►+), D (□►+):   Group of increase (n=14)
   E (+►□),   F (++►++), G (+►+):   Group of non-increase (n=11)

Fig. 1. Distribution of the examined samples with regard to the combined expression of topoisomerase I (i.e at the tumour's first appearance and at its recurrence)

6.1.2.3 Comparison with control group

No statistically significant differences were found concerning topoisomerase I expression in malignant cells from the primary tumour between patients in the study and the control group.

6.1.2.4 Association with other morphologic characteristics

With regard to tumour-adjacent morphologically non-dysplastic mucosa, when colonic crypts were cut longitudinally, some topoisomerase I positive colonic cells were detectable in the proliferation zone (lower 1/3 of the colonic crypts). In totally normal colonic mucosa, obtained from tumour-free surgical margins, no such expression was detectable.

6.1.3 Discussion

In the current study, 13 out of the 25 patients with colorectal cancer (52%) stained positive for topoisomerase I (Figure 2). In respect with the percentage of tumour cells staining positive for topoisomerase I, 9 out of the 25 patients (36%) presented with 5% - 50% positive staining cells and 4 out of the 25 (16%) with >50% positive staining cells. These data are in line with Boonsong et al. (Boonsong et al., 2002), while Staley et al. (Staley et al., 1999) demonstrated a higher percentage (86%) of positive staining in samples of 29 patients.

Topoisomerase I immunoreactivity was confined to be present in the nucleus of all biopsy samples, a finding which is consistent with the role of topoisomerase I as a nuclear protein.

Importantly, we displayed a significant increase of topoisomerase I expression by means of immunohistochemistry in recurrences of the initial neoplasia, thus reinforcing the notion that topoisomerase I expression is likely to be part of the malignant cells' phenotype in recurrent colorectal carcinomas. This is in agreement with previous experiments (Ichikawa et al., 1999; Paradiso et al., 2004), which demonstrated a direct correlation between thymidylate synthase (TS) and topoisomerase I expression in tumours and hypothesized, similarly to TS (Leichman et al., 1997), that high topoisomerase I expression is related to a more aggressive biological phenotype. However, our finding is in contrast with other studies (Boonsong et al., 2002; Paradiso et al., 2004), which have postulated the absence of a role for topoisomerase I in the acquisition of a metastatic phenotype. Such disperse results in the literature may reflect an interlesion heterogeneity concerning topoisomerase I expression.

Since the increased expression of topoisomerase I immunostain in neoplastic tissues from recurrences was demonstrated following 5-FU-based adjuvant chemotherapy regimen, it would be tempting to attribute this particular alteration to 5-FU itself. Additionally, given the fact that high levels of topoisomerase I expression has been shown to correlate with sensitivity to camptothecin chemotherapy (Monnin et al., 1999), patients with advanced colorectal cancer are likely to benefit from topoisomerase I-targeted anticancer drug therapy. This also might explain why patients with metastatic colorectal cancer appear to benefit more when they are treated with a combination chemotherapy regimen. Of note, strong synergism between 5-FU and irinotecan (CTP-11) has been reported (Guichard et al., 1997, 1998) after sequential exposure to both agents, whereas additivity or antagonism has been reported only after simultaneous exposure. The above facts are suggestive that the mechanisms of interaction between these two drugs might be multifactorial and the specific schedule of administration represents a critical parameter of their chemotherapeutic efficacy. In fact, the combination of CTP-11 and 5-FU+leucovorin (LV) has been approved (Saltz et al., 2000, 2001; Vanhoefer et al., 2001) as reference first-line chemotherapy for
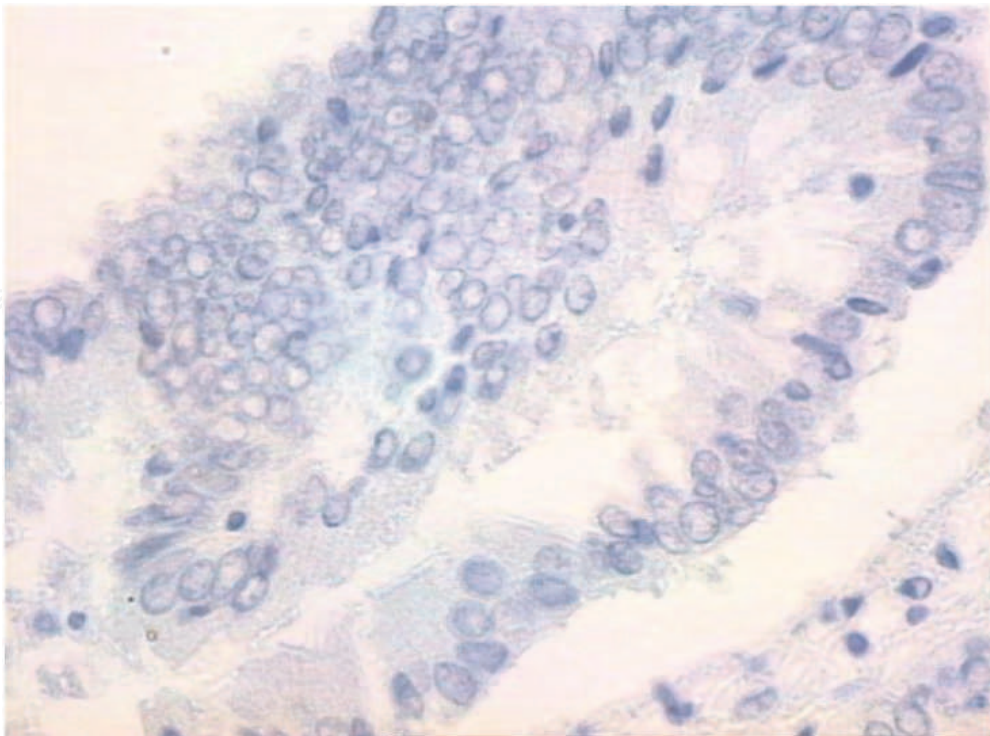
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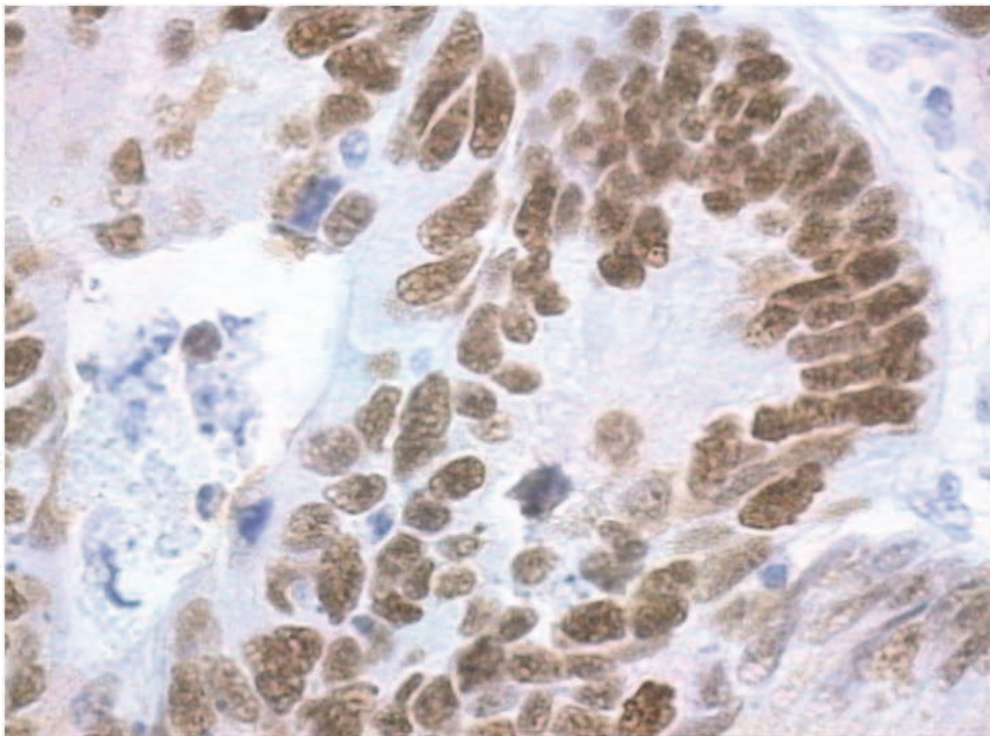
B

Fig. 2. (A) Absence of topoisomerase I staining in malignant cells of primary colorectal carcinoma (immunoperoxidase stain, x400) (B) Intense topoisomerase I reactivity, displaying a specific brown nuclear immunostaining, in malignant cells of the respective recurrence (immunoperoxidase stain, x400)

patients with metastatic colorectal cancer, a regimen being superior to 5-FU+LV alone which has been demonstrated to offer consistently improved tumour control and prolonged survival, albeit, only approximately 40% of advanced colorectal cancer patients seem to be potentially responsive to the aforementioned combination regimen (Waters et al., 2001).

One method of increasing the above described percentage of responsiveness to combination chemotherapy is relied on the availability of biomarkers, capable for identifying patients who might potentially respond to topoisomerase I inhibitor-based chemotherapy. With regard to CPT-11 efficacy, in vitro data have suggested that topoisomerase I expression could be considered as an important cellular sensitivity determinant (Paradiso et al., 2004). In particular, decreased DNA topoisomerase I expression has been shown to correlate with camptothecin resistant cell lines (Sanghani et al., 2003), a finding being in agreement with Jansen et al. (Jansen et al., 1997), who conversely reported a positive correlation between CPT-11 sensitivity and increased topoisomerase I activity. Other indicators for topoisomerase I drug response of colorectal cancer cell lines, possibly include a high growth fraction and a functional apoptotic pathway (Hafian et al., 2004), while Saltz et al. (Saltz et al., 1998) have suggested an association between CPT-11 efficacy and topoisomerase expression in colorectal neoplastic tissue; the latter being a potential predictor of 5-FU resistance (Leichman et al., 1997).

Moreover, in our study, the percentage or intensity of increase of topoisomerase I protein expression in recurrences did not demonstrate any significant correlations with Duke's stage, grade of tumour differentiation, localization, and patients' gender. These data suggest that there might be no obvious benefit from evaluating histologicaly the tumour-cell sensitivity to topoisomerase I-targeted drugs; subsequently, such drugs appear to be effective across a various spectrum of pathologies, stage and gender. However, the aforementioned increase of topoisomerase I expression was correlated with age, a fact which might be suggestive that treatment with topoisomerase I inhibitors would be more beneficial in older colorectal cancer patients. Of note, these data should be interpreted with caution due to the limited number of evaluated patients.

6.2 Main research on topoisomerase I and II expression in colorectal cancer cells
6.2.1 Patients and methods

6.2.1.1 Patients

Forty patients with colorectal cancer that had recurred following surgery and adjuvant chemotherapy who underwent a second operation were included in this study. All had undergone surgical resection of the primary tumour and had received post-operatively 5-FU-based [5FU and Leucovorin (LV), Mayo Clinic regimen] adjuvant chemotherapy (Heidelberger et al., 1957). Patients' characteristics are described in Table 1.

6.2.1.2 Study plan

The first tumour tissue was collected from the primary tumour during the initial operation, before the administration of any adjuvant chemotherapy. The second tissue sample was obtained at the time of recurrence, during the second operation and following chemotherapy. Both tissue samples for each patient, were analyzed for the expression of both topoisomerase-I and topoisomerase-IIa proteins.

Patients (*n*)	40
Gender	
Men	25
Women	15
Median age at diagnosis (years)	58 (35–75)
Performance status (WHO), 0–1	40
Location of primary tumor	
Right colon	6
Left colon	28
Rectum	6
Stage (Dukes')	
B2	13
C	27
Relapse	
Local	17
Distant	23
Differentiation	
Well	2
Moderate	31
Poor	7
Disease-free survival (months)	18 (range, 6–79)

Table 1. Patients characteristics

6.2.1.3 Determination of topoisomerase-I and topoisomerase-IIα

The expression of topoisomerase-I and topoisomerase-II*a* was studied on paraffin embedded biopsy sections using a standard three-step immunohistochemistry approach. Topogen, an anti-topoisomerase-I monoclonal antibody (mAb) (2012-3) was applied. Topogen is a mouse mAb (IgG2b isotype); its epitope has not been mapped (Libutti et al., 2005; Gouveris et al., 2007; Lazaris, 2002). The primary mAb JH2.7 for topoisomerase-II*a* (Biocare Medical, CA, USA) is a mouse mAb (IgG1 isotype) that recognizes a 170-kDa protein, the epitope of which maps between aminoacid 854–1447 of topoisomerase-II*a* (Gouveris et al., 2007; Lazaris et al., 2002; Zorzos et al., 2003]. Counterstaining with hematoxylin was applied at the end of the classical immunohistochemistry procedure.

6.2.1.4 Positive and negative controls

For the expression of topoisomerase-I and -IIα, normal human tonsil tissue immunostaining served as positive control samples. Nonspecific, isotype-matched monoclonal antibodies worked well as substitute negative controls (Gouveris et al., 2007; Lazaris et al., 2002; Libutti et al., 2005; Zorzos et al., 2003).

First step: Sections were examined for quantified immunoreactivity by two independent investigators blinded to any relevant patient clinical data. They evaluated more than 1,000 malignant cells in consecutive sections of neoplastic tissue specimens. The numbers of positive cells were then expressed as a percentage of labeled tumour cells with respect to the total number of tumour cells that could be idendified. Immunostaining for topoisomerase-I and -II*a* was then graded according to the percentage of tumour cells staining positively (- and ± for <5%, + for 5–50% and ++ for 50 to 75% of cells with positive staining). A characterization was given additionally for the intensity of topoisomerase immunostaining (weakly positive, moderately positive, or strongly positive). To simplify the scoring, we graded them as 0 (negative cells), 1 (± and/or <5%), 2 (+ and/or 5–50%), 3 (++ and/or 50–75%). Then, the biopsy specimens were analysed as paired samples: one section from the first surgery and one section from the second surgery for each patient.

Second step: The percentages of immunoreactive malignant cells were calculated using an image analysis system with an appropriate software package [Sigma Scan Pro, Version 5.0 (SPSS Science, Erkrath, Germany)]. The ratio was calculated as a percentage of immunohistochemically positive neoplastic cells over the total number (stained and unstained) of neoplastic cells. The membrane, cytoplasmic or nuclear intensity of the specific immunohistochemical stain, was also assessed. All the positively stained cells were classified for the purpose of simplicity into two groups: weakly stained and strongly stained.

DNA ploidy of neoplastic cells was evaluated by image DNA flow-cytometry performed on Feulgen-stained sections. Images were acquired using a Zeiss Axiolab microscope (Carl Zeiss Jena GmbH, Jena, Germany) with a mechanical stage, fitted with a SONY-iris CCD video camera (SONY Corporation, Tokyo, Japan). The latter was connected to a Pentium II personal computer, which included the relevant software. Slides were examined at low power magnification (40x) to identify the areas with the highest cellularity. In each case, a total number of ≥200 Feulgen-stained nuclei was selected at high power magnification (400x) and stored as JPEG file [1,550 x 1,070 pixels, 16.7 million colors (24-bit)]. Then the images were converted to gray scale and the staining intensity of the Feulgen-stained nuclei was measured semi-automatically. A classification of the nuclei in pairs according to their staining intensity followed. Finally, a graphic presentation of the nuclei, demonstrating their distribution according to their DNA content, was also performed.

6.2.1.5 Statistical analysis

All analyses were performed with SPSS version 10.1 (Statistical Product and Service Solutions; SPSS Inc., Chicago, IL, USA). We used McNemar's paired Chi-square test to assess the possible alteration of the levels of topoisomerases after chemotherapy with 5-FU. Fisher's exact test was performed in order to assess the possible relationship of topoisomerase increase with gender, Dukes' stage, tumour grade and localization. Mann–Whitney *U* Test was performed to assess a possible relationship between patient age and alteration in topoisomerases levels.

6.2.2 Results

6.2.2.1 Patients

Forty patients were included in the present study. Of these, 25 were males and 15 were females. The median age was 58 years (range 35 – 75). All patients had a performance status

(PS) of 0 – 1. Tumour localization at the time of diagnosis was: right colon in six patients, left colon in 28 patients, and rectum in the remaining six patients. In respect with pathological classification, 13 patients had Dukes B2 tumours, and the remaining 27 had Dukes C tumours. With regard to differentiation, two patients had well differentiated tumours, 31 patients had moderately differentiated tumours and seven patients had poorly differentiated tumours. Of all forty patients, 17 relapsed locally and 23 manifested with distant metastases at the time of recurrence. Overall, the median relapse-free interval was 18 (range 6 – 79) months (Table 1). All patients who entered the trial were finally evaluable for analysis.

6.2.2.2 Ploidy

Out of the 40 primary tumours, 12 were highly aneuploid and the remaining 28 were moderately aneuploid. There was no association between the degree of DNA aneuploidy and the expression of any of the analyzed markers.

6.2.2.3 Topoisomerase I

Immunohistochemical analysis revealed that levels of topoisomerase-I expression were higher in malignant cells from tumour recurrences compared to cells from the primary tumours (2 sided paired Chi square test, $P = 0.0001$) (Table 2; Fig. 1). Topoisomerase-I expression was also decreased in grade 1 ($P = 0.007$) and increased in grade 3 tumours (2 sided Fischer's exact test, $P = 0.003$) (Table 2). By image analysis evaluation, a significant raise in malignant cells from the tumour recurrences could be recognised (2 sided paired Chi square test, $P = 0.0001$) (Table 3); low expression of topoisomerase-I was noticed in range 1–5 ($P = 0.0001$) and 26–30 (2 sided Fischer's exact test, $P = 0.0143$) (Table 3; Fig. 3). The increase in topoisomerase-I levels was not significantly correlated with gender, performance status (WHO), location of the primary tumour, Dukes' stage, grade of differentiation, and localization of relapse. There was a statistically significant correlation between the age of patients and the expression of topoisomerase-I (Mann–Whitney U Test, $P = 0.011$). Patients with an increased expression of topoisomerase-I levels were older in age (median=62.5 years) than patients without increased expression (median=50 years).

6.2.2.4 Topoisomerase IIα

Malignant cells from tumour recurrences showed a statistically significant increase of the levels of topoisomerase-II, compared to those of the primary tumours (2 sided paired Chi square test, $P = 0.0001$) (Table 2; Fig. 2). The topoisomerase-II levels were decreased in grade 3 lesions (2 sided Fischer's exact test, $P = 0.0001$) (Table 2; Fig. 4). In image analysis, a significant increase in malignant cells from tumour recurrences could be identified (2 sided Fischer's exact test, $P = 0.0001$) (Table 3); low expression of topoisomerase-II was noticed in the range of 16–20 (2 sided Fischer's exact test, $P = 0.0143$) (Table 3). Levels of topoisomerase-IIa expression were also higher in malignant cells from tumour recurrences compared to cells from primary tumours (Chi square test, $P = 0.0001$). There was a statistically significant positive correlation between the age of patients and increased levels of expression of topoisomerase-IIa (Mann–Whitney U Test, $P = 0.011$). The increase in topoisomerase-IIa levels did not demonstrate any significant correlation with gender, performance status (WHO), localization of primary tumour, Dukes' stage, tumour grade, and location of relapse, nor were such correlations found between the above parameters and the differences of topoisomerase-IIa levels in the primary tumours and relapses.

Examined parameters	Photo, microscopic evaluation			P
	Grade	First evaluation	Second evaluation	
Topoisomerase I	0	7	0	0.0117
	1	13	1	0.0007
	2	17	26	0.0722
	3	3	13	0.0103
Increased: 25, decreased: 0, no change: 15, $P = 0.0001$				
Topoisomerase IIa	0	0	0	1
	1	10	2	0.0252
	2	30	28	0.8027
	3	0	10	0.0010
Increased: 18, decreased: 0, no change: 22, $P = 0.0001$				

Table 2. Photomicroscopic evalution

6.2.3 Discussion

In our study, 33 out of 40 (83%) patients had biopsy samples obtained from colorectal cancer tissue that stained positive for topoisomerase (Table 2; Fig. 3). This finding is in close agreement with the results of a study by Staley et al. (Staley et al., 1999), who reported 86% of positive staining in a total of 29 samples, but in contrast with the results reported by Boonsong et al. (Boonsong et al., 2002), who demonstrated a lower percentage of cells expressing topoisomerase-I: 51% of the samples, including 24.4% with >50% positive tumour cells (Boonsong et al., 2002). Topoisomerase-I immunoreactivity in our study was confined to the nucleus in all samples, being consistent with the role of topoisomerase-I as a nuclear protein. For topoisomerase-IIa, 21 out of the 40 samples (52%) of colorectal cancer tissue stained positive (Table 2; Fig. 4). Half of them, i.e. 10/40 (25%) presented only weak positive staining for topoisomerase IIα (grade 1), a finding confirmed by Image Analysis (Table 3). The above data are consistent with those reported by Burden and Osheroff (Burden et al., 1998).

In the present study, we reported a significant increase in topoisomerase-I and topoisomerase-IIa expression in tissues from recurrent colorectal tumours, reinforcing the view that the expression of topoisomerases is likely part of the malignant-cell phenotype in colorectal carcinomas that relapse after initial treatment. This observation is in agreement with previous studies (Paradiso et al., 2004; Tsavaris et al., 2004), which demonstrated a direct correlation between thymidylate synthase (TS) and topoisomerase-I tumour expression and hypothesized, similar to TS (Ichikawa et al., 1999), that high topoisomerase-I expression is related to a more aggressive biological phenotype. Of note, increased topoisomerase-IIa expression characterizes rapidly proliferating cells (Burden et al., 1998) and may represent a useful marker of aggressive tumour behavior (Leichman et al., 1997).

Since the increase of topoisomerase-I and -IIa expression was demonstrated following 5-FU-based adjuvant chemotherapy therapy, it would be tempting to attribute this change to 5-FU itself. High levels of topoisomerase-I correlate with sensitivity to camptothecins (irinotecan and topotecan), as it has been shown in the litterature (Monnin et al., 1999), whereas high levels of topoisomerase-IIa correlate with sensitivity to etoposide, a well-established topoisomerase-IIa-targeting agent (Leichman et al., 1997). Thus, it would be reasonable to

consider administering these agents sequentially in a regimen used for the treatment of colorectal cancer, i.e. 5-FU → irinotecan and 5-FU → etoposide.
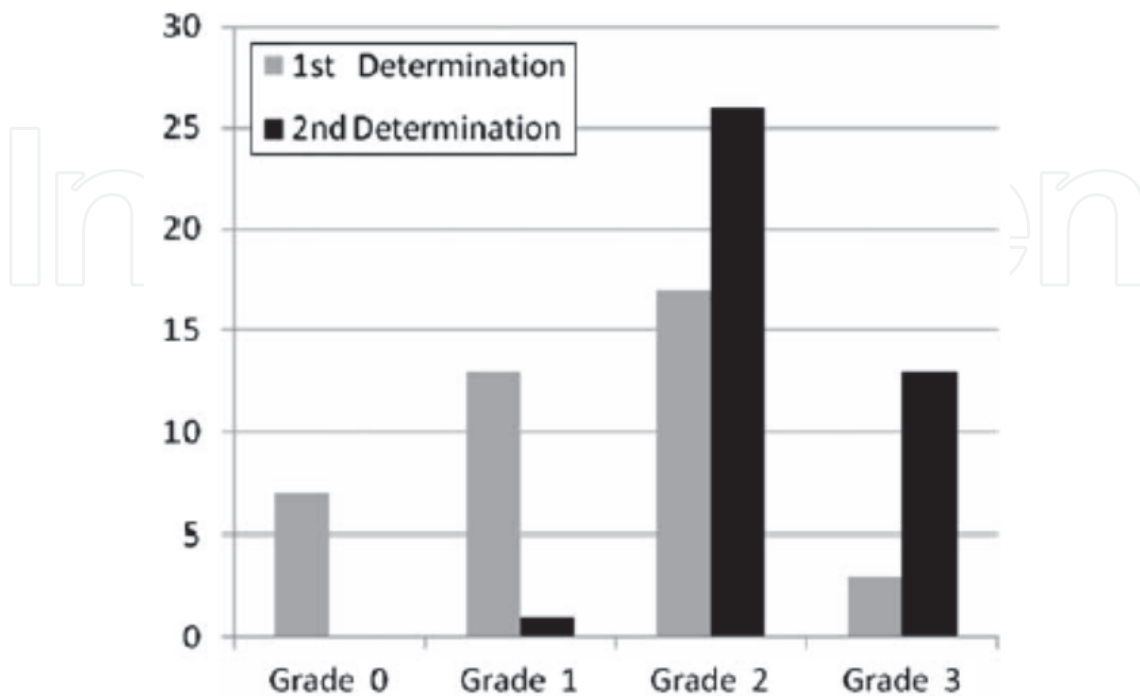

Fig. 1. Topoisomerase I (Topo-I) expression in relation to tumour grade according to immunohistochemical evaluation
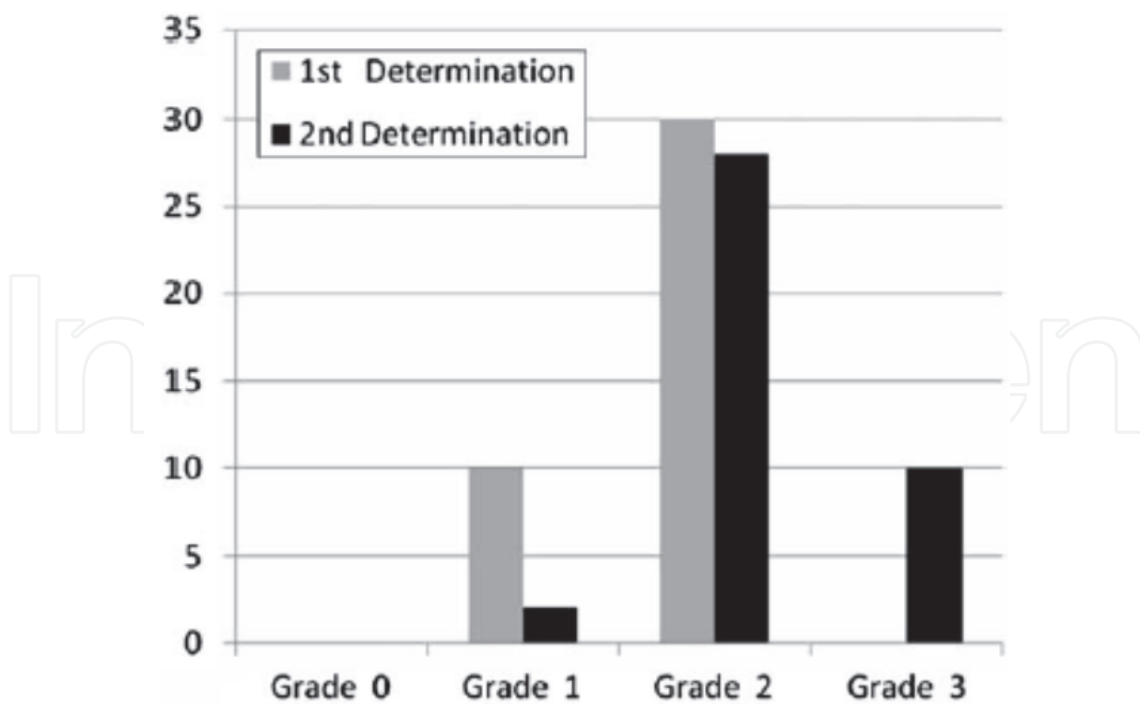

Fig. 2. Topoisomerase IIa (Topo-IIa) expression in relation to tumour grade according to immunohistochemical evaluation

Irinotecan (CPT-11) is a water soluble camptothecin derivative, which acts as a specific and potent inhibitor of topoisomerase-I (Hande et al., 1998). In a European-originated phase II study, irinotecan achieved response rates of 19% in chemotherapy-naive patients with colorectal cancer and 18% in chemotherapy pre-treated patients with advanced disease (Luzzio et al., 1995).

Irinotecan yielded response rates of 15–25% in colorectal cancer patients refractory to 5-FU-based treatments (Bognel et al., 1995; Conti & Saltz, 1994; Gennatas et al., 2006; Gupta et al., 1994; Pitot & O'Connell, 1994; Rothenberg & Burris, 1994; Tsavaris et al., 2003, 2007; Wilstermann et al., 2007), not dissimilar to those observed in first-line treatment of colorectal cancer patients (18–32%) (Mitry et al., 1998; Tsavaris NB et al., 2002; Van Custen & Peeters, 1998) and in contrast to the expected decreasing response rate from the application of irinotecan as a second-line treatment agent. Treating patients with irinotecan upon progression after chemotherapy with 5-FU+LV seems to be yielding more signiffcant results compared to the opposite sequence, based on our previous experience, where the best results with sequential monotherapies were obtained when 5-FU+LV was followed by irinotecan (5-FU/LV → CPT11) at disease progression or relapse (Gupta et al., 1994).

Range	First sample (no.)	Second sample (no.)	P	Range	First sample (no.)	Second sample (no.)	P
Topoisomerase I							
0	0	0	1	36–40	3	0	0.2405
1–5	21	1	0.0001	41–45	1	1	1.000
6–10	4	0	0.1156	46–50	1	1	1.000
11–15	2	4	0.6752	51–55	2	4	0.6752
16–20	3	5	0.7119	56–60	0	4	0.1156
21–25	2	3	1	61–65	0	5	0.0547
26–30	1	9	0.0143	66–70	0	0	
31–35	0	3	0.2405	71–80	0	0	
Topoisomerase IIa							
0	0	0	1	36–40	2	3	1
1–5	10	4	0.1395	41–45	5	1	0.2007
6–10	6	5	1	46–50	0	3	0.2405
11–15	5	2	0.4315	51–55	0	2	0.4937
16–20	9	1	0.0143	56–60	0	0	1
21–25	0	5	0.0547	61–65	0	1	1
26–30	3	3	1	66–70	0	2	0.4937
31–35	0	3	0.2405	71–80	0	5	0.0547

Total	First determination			Second determination			P
	Mean	Median	Range	Mean	Median	Range	
Topoisomerase I	13.875	4.5	1–54	35.375	29.5	4–65	0.0001
Topoisomerase IIa	16.775	15	1–45	34.575	31	2–79	0.0001

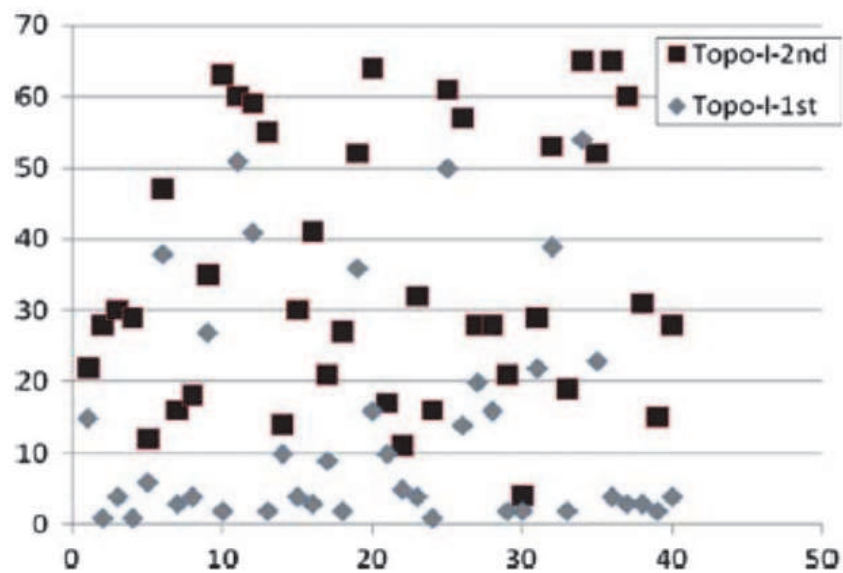Table 3. Image analysis evalution (%)

Fig. 3. Dispersion diagram of topoisomerase I (Topo-I) expression as assessed by immunohistochemistry (x axon are the number of cases analysed and the y axon represents the percentage of cells expressing topoisomerase I in first and recurrent tumour). It illustrates the increased levels of topoisomerase I in recurrences.

With regard to irinotecan efficacy, in vitro data suggest that topoisomerase-I expression could be regarded as an important cellular sensitivity determinant (Coleman et al., 2000). In particular, decreased DNA topoisomerase-I expression was correlated with camptothecin-resistant cell lines (Gouveris et al., 2007), while another study (Wilstermann et al., 2007) reported a positive correlation between irinotecan sensitivity and topoisomerae-I activity.
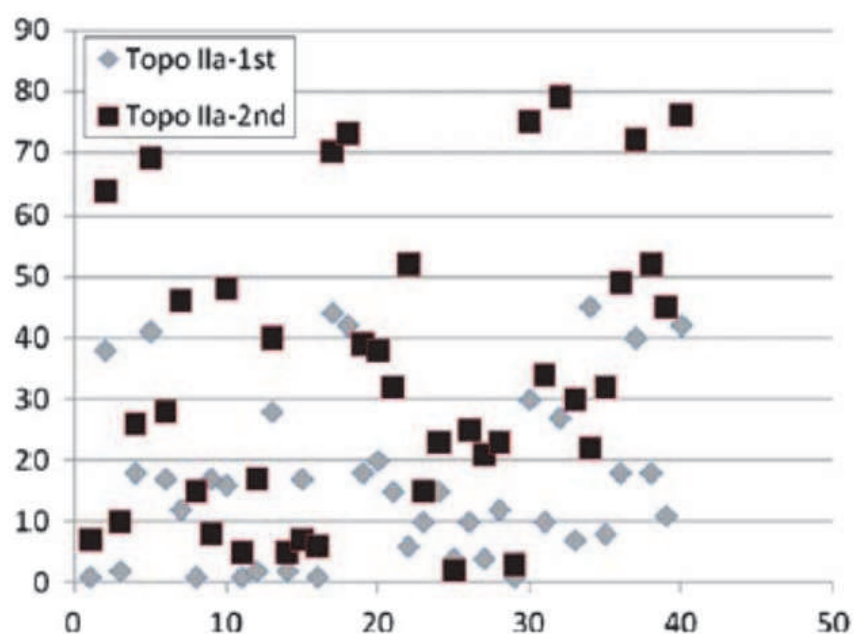

Fig. 4. Dispersion diagram of topoisomerase II*a* (Topo-II*a*) expression as assessed by immunohistochemistry (x axon are the number of cases analysed and the y axon represents the percentage of cells expressing topoisomerase II in first and recurrent tumour). It illustrates the increased levels of topoisomerase II in recurrences

Drugs that target topoisomerase-II, such as the epipodophylotoxins, etoposide (VP-16) and teniposide (VM-26) (Leichman et al., 1997) (VP-16), doxorubicin, and mitoxantrone, are among the most effective anticancer drugs still in clinical use. Especially, etoposide acts by destroying cells via inhibition of the ability of topoisomerase-II to ligate nucleic acids which are cleaved during the double-stranded DNA passage reaction (Bleiberg, 1998). Studies including previously untreated patients with advanced colorectal carcinoma evaluating the combination of etoposide with cisplatin or 5-FU demonstrated minimal activity in metastatic colorectal cancer (Boige et al., 1998; Passalacqua et al., 1991; Planting et al., 1996; Posner et al., 1990). There have been no clinical data supporting the in vitro synergy observed between these cytotoxic agents (Colucci et al., 1995; Zelkowitz et al., 1989). Other studies failed to prove any benefit with the combination of etoposide with 5-FU or cisplatin/carboplatin (Inaba et al., 1994). However, the combination of etoposide with 5-FU + LV had demonstrated some activity when administered as second-line treatment after failure of weekly 5-FU + LV in patients with metastatic colorectal cancer (Stuart et al., 1995; Tsavaris et al., 2002).

The results reported herein underscore the role of topoisomerases (topoisomerase-I and topoisomerase-II*a*) expression in colorectal cancer. It is believed that translational studies of molecular targets for currently applied cytotoxic and biological agents (like bevacizumab and cetuximab) might form the basis of shaping current and future drug combinations and of rationalizing the optimal chemotherapeutic drug schedule and sequence, which will eventually translate in improved tumour eradication and prolongation of survival.

This work is unique since it presents the first two of a series of studies that demonstrate an increase of topoisomerase expression following chemotherapy with 5FU. This may denote that such tumours are sensitized through 5FU chemotherapy regimens to topoisomerase inhibitors, providing a pathophysiologic mechanism to explain the described effectiveness of such agents in recurrent colorectal cancer after the first adjuvant chemotherapy.

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The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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