

## **Papel de las metaloproteinasas en los tumores digestivos**

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Las células epiteliales, cuya transformación causa el 90% de los cánceres en el hombre adulto, se caracterizan por la formación de estrechas uniones intercelulares y su asociación a la matriz extracelular que frecuentemente se sitúa subyacente a ellas. Hoy está generalmente aceptado que la formación y la preservación adecuada de las uniones intercelulares es imprescindible para el mantenimiento de la diferenciación de las células epiteliales y que su desestabilización facilita la desdiferenciación y adquisición de la capacidad invasiva, con la consiguiente aparición de carcinomas. Los procesos de invasión tumoral y metástasis involucran una compleja cascada de acontecimientos que incluyen el crecimiento tumoral y la angiogénesis, la invasión local, procesos de intra y extravasación y finalmente el crecimiento y desarrollo del proceso tumorigénico en localizaciones mas o menos alejadas del sitio donde se desarrolló el tumor primario. En cada estadio de la cascada metastásica tienen lugar una serie de interacciones, complejas y coordinadas, entre el tumor y el tejido que lo rodea (matriz extracelular), las cuales involucran la participación de proteasas, inhibidores de proteasas y moléculas de adhesión. La invasividad incluye varias etapas, a saber: pérdida de la adhesión intracelular, degradación de la matriz extracelular y movilidad. Todo ello permite a las células tumorales infiltrarse en el tejido que las rodea, al mismo tiempo que siguen conservando una elevada capacidad para multiplicarse.

La degradación de la matriz extracelular, segunda etapa del proceso de invasividad, está basada en el aumento de la actividad proteolítica. Las proteasas que están relacionadas con este proceso degradativo, tanto en situaciones normales como patológicas, son las metaloproteasas de matriz extracelular (MMPs). En esta familia de proteínas se incluyen mas de 20 miembros, que se clasifican en distintos subgrupos en función de los sustratos sobre los que actúan de forma preferente, y también atendiendo a los dominios que aparecen en la estructura de las mismas. De esta forma, los grupos principales incluyen a las colagenasas, que actúan degradando las fibras de colágeno; las gelatinasas, que son metaloproteasas con elevada actividad sobre el colágeno tipo IV y V; las estromelisin, que degradan componentes de tipo “no colágeno” de la matriz extracelular, y las metaloproteasas de membrana (MT-MMPs), que son moléculas de esta familia con dominios transmembrana. Las MMPs se piensa que tienen una importante función durante el desarrollo y la fisiología normal del organismo, permitiendo la migración celular o modulando la actividad de ciertas moléculas por procesos proteolíticos.

La regulación de su actividad “in vivo”, en condiciones normales, se encuentra estrechamente controlada a varios niveles: activación de la expresión génica, activación extracelular de las MMPs secretadas en forma latente, y procesos de inhibición en los que participan inhibidores tisulares de las metaloproteasas de matriz (TIMPs). En ciertos procesos patológicos, entre los que se incluye el cáncer, las MMPs no están normalmente reguladas y el balance entre formas activas e inactivas favorece la proteólisis. Al estar estas proteínas relacionadas con la degradación de la matriz extracelular, con la importancia que este hecho presenta en la progresión de los acontecimientos relacionados con la cascada metastásica, los niveles de MMPs activas pueden considerarse como factores pronóstico de importancia en el cáncer. Un ejemplo de gran interés conceptual y práctico lo constituye el carcinoma colorrectal. Los tumores de colon derecho, desdiferenciados y aneuploides, presentan un mal pronóstico tras cirugía. De hecho la supervivencia global de los mismos no supera el dintel del 60%. Con relación al cáncer colorrectal se han desarrollado, en los últimos años, diversos estudios encaminados a la detección de niveles de distintas metaloproteasas y/o sus inhibidores. Sin embargo, la mayor parte de estos estudios han sido llevados a cabo en un escaso número de muestras y empleando metodologías dispares, por lo cual los resultados obtenidos hasta la fecha no son concluyentes, aunque sí parece existir una correlación positiva entre los niveles de metaloproteasas activas y la progresión tumoral. Sin embargo, los tumores colorrectales de colon izquierdo, diferenciados y euploides, presentan un mejor pronóstico tras cirugía curativa. Dichos tumores presentan inestabilidad a microsatélites y fenotipo mutador (RER+). Una atractiva hipótesis consistiría en que dichos tumores presenten mejor pronóstico debido a una menor capacidad de inducción de metaloproteasas invasivas.

Además del cáncer colorrectal, recientemente, el modelo carcinogénico del fenotipo mutador ha sido asociado con otros tumores digestivos tales como el cáncer gástrico. Si bien el número de trabajos de investigación en los que se consideran estos otros procesos tumorales es considerablemente más reducido que los existentes para el cáncer colorrectal, datos recientes parecen indicar que también los carcinomas gástricos con inestabilidad en microsatélites tienden a conferir un pronóstico favorable a los pacientes afectados. Concretamente, en tumores de estómago, se han detectado diferencias significativas en la supervivencia de los pacientes que desarrollan tumores con inestabilidad en microsatélites, en relación con aquellos que presentan tumores estables para esta alteración, siendo el primer grupo de individuos los que manifiestan un riesgo más reducido de desarrollar recidivas después de la resección quirúrgica del tumor primario. De hecho, hay hipótesis que indican que las diferencias en la supervivencia de ambos grupos de

pacientes serían el resultado de una tendencia más elevada a generar metástasis en el caso de los tumores sin inestabilidad en microsatélites y que, por tanto, no cursan por la vía del fenotipo mutador.

Sin embargo, en cualquiera de los casos descritos, se desconoce el mecanismo molecular responsable del diferente comportamiento clínico de los carcinomas que se desarrollan a través de la vía del fenotipo mutador.

## **Determinants of prognosis and response to therapy in colorectal cancer**

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### **Introduction**

In 1994, there were an estimated 159,000 new cases of colorectal cancer diagnosed in the United States and 56,000 deaths from this disease (1,2). Colorectal cancer is the third most common cause of cancer-related death in men and women. Although there is no strong gender effect in colorectal cancer incidence or mortality, colorectal cancer is the leading cause of cancer mortality in women 75 years old and above (1).

A major problem in cancer pharmacology is the prediction of the outcome of therapy, both in terms of tumor response and host toxicity. Pharmacogenetic variability in drug metabolizing enzyme systems is a major determinant of variations in these outcomes. Unpredictable disposition of drugs may result in an undertreatment failing to provide therapeutic effects, or an overtreatment leading to excessive toxicity. It is important for clinical researchers to identify and investigate therapeutic agents that exhibit unpredictable toxicity and variable pharmacokinetics/pharmacodynamics, which may be genetically determined. Pharmacogenetic screening prior to chemotherapy will enable the identification of patients who may be deficient in a critical detoxifying enzyme as shown for DPD or a decreased activity of TS and TP in patients treated with fluoropyrimidines.

Cancer chemotherapy is limited by significant inter-individual variations in responses and toxicities. Such variations are often due to genetic alterations in drug metabolizing enzymes and/or drug target gene expression. Screening for molecular predictors prior to anticancer drug administration may lead to identification of specific populations predisposed to drug toxicity and/or poor drug response. The significance of molecular pharmacogenetics in cancer chemotherapy is critical due to the following reasons.

1. Anticancer agents generally have a narrow margin of safety
2. Many of these genes are drug targets and are biotransformed to active compounds by enzymes that exhibit genetic polymorphism or are expressed differentially in tumors versus normal tissue
3. Certain anticancer drugs are detoxified by polymorphic enzyme systems
4. Most cancer drugs have significant inter-patient variability in pharmacokinetics and toxicity

### **Fluoropyrimidines: Molecular predictors of response and survival**

Fluoropyrimidines (FP) are an important group of antineoplastic drugs that are widely used in the treatment of gastrointestinal tumors (3,4). A major mechanism of action of these drugs is the inhibition of thymidylate synthase (TS). This enzyme, the only *de novo* source of thymidine in cells, is essential for DNA synthesis and, therefore, also limiting for cell growth. The sensitivity of tumor cells to FP's depends on effective TS inhibition (5,6,7). Indeed, one mechanism by which cells acquire resistance to 5-FU is gene amplification (5,6,7). We have previously shown that variation in basal TS gene expression levels among patients' tumors is a statistically significant predictor for response to 5-FU. Those tumors with high TS expression are generally non-responsive to protocols that include the TS-directed combination of 5-FU and leucovorin (LV) (8-12). Moreover, we have demonstrated the association between TS protein and TS gene expression, and their inverse relationship to response in metastatic colorectal and primary gastric cancer (10). However, while high TS expression levels effectively predict for non-response, low TS expression did not necessarily predict response: all responding patients had low TS expression, but a subset of patients with low TS expression levels did not respond to treatment (8). These non-responders therefore may have other mechanisms of resistance that the favorable condition of low TS is insufficient to overcome.

Capecitabine is a new orally-available tumor selective fluoropyrimidine carbamate, designed to generate 5-FU selectively in tumors. Capecitabine is converted to 5-FU by three enzymes located in the liver and tumors, the final step is the conversion of 5'-dFUrd to 5-FU by thymidine phosphorylase in tumors (13,14). This higher therapeutic index appears directly related to the tumor-specific generation of 5-FU by the tumor associated enzyme thymidine phosphorylase (15,16). In clinical studies the 5-FU levels were significantly higher in primary colorectal tumor tissues than in adjacent normal tissues possibly explained by the 4 fold difference in TP activity between primary colorectal tumors and healthy colorectal mucosa was close to the ratio of 5-FU in the primary tumor compared to healthy tissue (Roche Product Information). Our data suggest that TP gene expression is an independent predictor of response to 5-FU based chemotherapy.

In addition to TS protein or RNA expression in tumor tissues, TS gene polymorphisms may determine response to therapy. The human thymidylate synthase gene promoter is polymorphic, having either double or triple tandem repeats of a 28 base-pair sequence. The reporter gene linked to the promoter region of the TS gene with triple tandem repeats had 2.6 fold higher expression activity when compared to double tandem repeats. Previously we

described patients with colon cancer homozygous for the triple tandem repeat variant had 3.5 times higher TS mRNA level when compared to those homozygous for the double repeat variant. When tested, pts with disseminated colon cancer treated with 5-FU, pts homozygous for the double tandem genotype had a significantly higher response rate when compared with those with the triple tandem genotype, and a mixed response for pts heterozygous. Thus providing a means of selecting patients with the TS polymorphism may provide opportunity to optimize therapy in patients with tumors likely to respond (22). Recent data suggest that TS polymorphism is also predicting response and toxicity to Xeloda chemotherapy (in press).

Low expression of TS expression does not predict 100% response but increases the probability of response to 5-FU based therapy. Therefore, Danenberg et al have looked for other molecular determinants of response and survival in patients treated with 5-FU based chemotherapy. In addition to TS, they demonstrated that gene expression levels of thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) are independent predictors of response to 5-FU(23).

This has recently been supported by data we published in which we have evaluated DPD expression, measured by quantitative RT-PCR, in 33 pretreatment biopsies of colorectal tumors from patients who went on to receive treatment with 5-FU based therapy. The range of gene expression of DPD for responders ( $0.60 \times 10^{-3}$  to  $2.5 \times 10^{-3}$ , 4.2 fold) was relatively narrow compared with that of the nonresponders ( $0.2 \times 10^{-3}$  to  $16 \times 10^{-3}$ , 80 fold). The difference in gene expression between responding and nonresponding groups was statistically significant, as was the lack of responding tumors with DPD expressions  $>2.5 \times 10^{-3}$ . There was no correlation among DPD, TS, and TP expression values in these tumors, indicating that these gene expressions are independent variables. Tumors that responded to 5-FU therapy had expression values of all 3 genes, TS, TP, and DPD below the nonresponse cutoff values, this group of patients had a 92% response rate(23). Nonresponding tumors had at least one of these values for gene expression that was high. Those patients with low expression of all three genes had significantly longer survival than patients with a high value of any one of these gene expression levels. Low intratumoral DPD levels predict a response to 5-FU chemotherapy, but the use of more than one determinant of response, that is TS and TP expression, allows the identification of a high percentage of responding patients

Activity of DPD may be determined by measuring enzymatic activity or mRNA levels. A recent study has revealed a linear relationship between DPD activity and DPD mRNA levels in tumor. Thus revealing that mRNA levels by RT-PCR reflects the DPD activity of colorectal

tumor tissue, in turn allowing prediction of response to 5-FU chemotherapy. Also of note is that DPD activity in normal mucosa was found to be significantly higher than that in tumor tissue(33).

The capability of an almost absolute prediction of non-response as well as identification of a set of patients with very high but not absolute probability of response has significant impact on the design of new treatment regimens with fluoropyrimidines. Tumors with high TS, TP and DPD expression levels should be treated with non-TS directed anticancer drugs such as CPT-11 or oxaliplatin, or in combination with 5-FU. We have some understanding that molecular determinants play an important role in response to 5-FU. With the development of new effective anticancer drugs such as CPT-11 and oxaliplatin, it is of clinical significance to better understand the metabolism and the mechanism of resistance of these new active agents. It is essential to understand why some patients develop life-threatening toxicity and why some tumors are resistant to CPT-11 or oxaliplatin. We have identified potential molecular determinants of the metabolism of CPT-11 and oxaliplatin and molecular predictors of response to these agents.

Oxaliplatin is a new platinum analog of the DACH family (51). A high response rate (28-65%) with the triple association (FU/folinic acid/oxaliplatin) has been reported in advanced colon cancer treated in first and second line settings indicating some synergistic effect with fluoropyrimidines (colorectal) (51,52). Oxaliplatin cytotoxicity was significantly superior to cisplatin in cell lines with both acquired (H12DDP) and intrinsic (1777NRp Cl-A) intermediate level resistance to cisplatin. The dissimilarity in profiles of oxaliplatin and cisplatin suggests that these two platinum compounds have a different target(s)/mechanism(s) of action, a different mechanism(s) of resistance, or most likely both. Oxaliplatin has a different spectrum of activity and low cross-resistance to cisplatin and should be valuable in cisplatin refractory patients. We have established molecular predictors of response to cisplatin therapy. We have shown that ERCC-1 gene expression levels predict response and survival in patients with primary gastric cancer treated with 5FU/cisplatin therapy. ERCC-1 gene expression was independent from TS gene expression levels (53). Preliminary data suggest that TS and ERCC1 may be predictors of response to 5FU/oxaliplatin therapy (JCO in press). In addition, we have shown that genomic polymorphism of DNA repair enzymes such as XRCC-1, XPD and GST-P1 are associated with clinical outcome in patients with metastatic colorectal cancer treated with 5-FU/oxaliplatin in second or third line chemotherapy (Cancer Research in press and unpublished data)

## Conclusions

Our goals are: 1) to tailor chemotherapy based on the molecular profile of an individual tumor and the individual genetic background of patients with colorectal cancer; 2) to decrease mortality of patients with colorectal cancer with more efficient and less toxic treatment based on the molecular profile of the tumor and genetic properties of the patient; 3) to identify novel targets of tumor resistance or tumor recurrence to allow the development of new anticancer drugs and potential gene replacement strategies.

Our conclusions based on the data presented are:

1. Gene expression levels can be accurately measured in tumor biopsy specimens.
2. Gene expressions can be identified that are predictive for the in vivo antitumor activity of drugs.
3. The use of these determinants to plan the treatment for each patient should increase the effectiveness of chemotherapy.
4. Genomic polymorphisms of gene involved in drug metabolism may predict efficacy and toxicity of chemotherapy.
5. Molecular profiling may allow a more efficient monitoring and individualized surveillance.

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## **Presents and future of new drugs designed for new treatment targets**

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Chemotherapy is not expected to further increase the chances of cure or improve palliative care in patients with cancer. Several alternative targets are found to be involved in the development of cancer cells and identified as potential targets for new treatment options.

In the complex process of tumor development and metastases formation the development of new blood vessel and degradation of extracellular matrix are believed to be fundamental steps. Angiogenesis is a physiological process and highly regulated by a family of pro- and antiangiogenic factors. Some of these agents, such as vascular endothelial growth factor (VEGF) are highly specific for the endothelial compartment, whereas others, such as matrix metalloproteinases, have various nonangiogenic activities. Recently, "dormancy" of micrometastatic deposits is found to be similarly dependent on angiogenesis and that a balanced rate of proliferation and apoptosis can be tipped in favor of cell growth when the tumor evolves an angiogenic phenotype.

Recombinant human monoclonal antibody against VEGF (rhMAB VEGF) blocks proliferation and migration of vascular endothelial cells. Preoperative serum VEGF was found to be associated with tumor stage in patients with colorectal cancer. Vessel count, VEGF and PD-ECCF correlate with metastasis in human colon cancer. The blood cells of cancer patients contain several fold higher concentrations of VEGF than those of healthy control patients. VEGF is probably transported in the blood stream by blood cells including leukocytes and platelets.

Data on safety for rhMAB-VEGF in men are available and indicate to be well tolerated. Interestingly, early phase II data in combination with 5-FU/LV based chemotherapy indicate improved antineoplastic activity. This treatment option is currently further investigated in phase III trials.

Potential targets of new drugs also include the matrix metalloproteinases (MMPs), a group of proteinases that have physiological roles in degrading and remodeling extracellular matrix. MMPs are overexpressed in a variety of malignant tumor types and their overexpression is associated with tumor aggressiveness and metastatic potential. MMPs fall into five classes

according to their primary structure and substrate specificity: collagenases, gelatinases, stromelysins, membrane type and nonclassified. Several MMP inhibitors are under clinical development that differ in their specificity against MMPs and class. In phase I/II trials some agents have dose limiting musculoskeletal side effects such as inflammatory polyarthritis that typically appeared during the first month of treatment. Also, liver dysfunction and thrombopenia have been observed.

BAY12-9566 has been evaluated in phase II clinical trials in patients with pancreatic, ovarian, and lung cancers, but studies have reported disappointing results.

The Ras family proteins are mutationally activated in a wide range of human tumor types and are important contributors to the neoplastic phenotype. Three ras proto-oncogenes have been identified: H-ras, K-ras and N-ras genes. Ras functions as a molecular switch that cycles between an inactive GDP bound stage and an active GTP bound stage. Ras is localized to the inner surface of the plasma membrane only after it has undergone post-translational modification by farnesylation, the first and most critical step. Farnesyltransferase inhibitors are either CAXX mimetics, analogs, bisubstrate inhibitors or inhibitors identified from compound libraries. R115777 is the first inhibitor to be studied in clinical trials, which can be given orally. Other compounds are SCH66336 and BMS214662. All are in clinical trials but most of them have not demonstrated major activity. R15777 had dose limiting neuropathy and also nausea, vomiting, headache, fatigue, anemia and hypotension. Phase I trials of FFTs in combination with 5-FU/LV, Gemcitabine, Cisplatin are reported.

The epidermal growth factor receptor (EGFR) autocrine pathway contributes to a number of processes important to cancer development, including cell proliferation, apoptosis, angiogenesis and metastatic spread. Activation of the TGF $\alpha$ -EGFR pathway can be attributed to several mechanisms, such as overexpression of the EGFR, increased concentration of ligands, decreased phosphatase activity, decreased receptor turnover, and the presence of aberrant receptors including EGF gene alterations such as the mutant found in EGFRvIII that has a constitutively activated tyrosine kinase domain that stimulates cell proliferation independently of ligand activation. Inhibition of EGFR signaling in cancer therapy may be achieved by Mabs or small molecule inhibitors of the EGFR tyrosine kinase. IMC-C225 is a MAb with promising activity in patient with 5-FU and CPT-11 resistant colorectal cancer and other tumor types such as head and neck cancer. Clinical trials are

currently ongoing. EGFR MAb or the small molecules have acneform skin side effects that can resolve although the treatment is continued. Some of these compounds also induce diarrhea, which may be important for combination with certain antineoplastic drugs. Examples of small molecule inhibitors are ZD1839 (Iressa), OSI-774, PKI-166 and EKB-569 and others that are in different steps of their clinical development. ZD1839 has demonstrated interesting activity in NCSLC and will be further investigated by the EORTC GI-groups in several phase I trials.

Epidemiological studies have documented a 40-50% reduction in the incidence of colorectal cancer in individuals taking nonsteroidal antiinflammatory drugs (NSAIDs), which is believed to mainly contribute to inhibition of cyclooxygenase (COX) enzyme activity. NSAIDs inhibit both COX-1 and COX-2. The more selective COX-2 inhibitors are associated with fewer gastrointestinal side effects. Data indicate a crucial role for COX-2 in colon carcinogenesis. The conversion of arachnoidic acid to prostaglandins is catalyzed by the COX family of enzymes. Aspirin and sulindac inhibit both COX-1 and COX-2, whereas celecoxib and rofecoxib inhibit only COX-2. These agents induce apoptosis by both COX-dependent and COX-independent mechanisms. The inhibition of COX-2 leads to an increase in arachnoidic acid, which, in turn, stimulates the conversion of sphingomyelins to ceramide, a mediator of apoptosis. Inhibition of COX-2 may also lead to apoptosis by altering prostaglandin production and by decreasing angiogenic factors. Clinical studies of patients with familial adenomatous polyposis indicate decrease in number of polyps by sulindac and celecoxib and may be interesting agents for adjuvant treatment of colorectal cancer. Also, preclinical models indicate single agent activity in some tumor model systems and synergistic activity with radiation and antineoplastic agents such as 5-FU and CPT-11. The MRC, PETACC initiative and the EORTC GI group are discussing the possibility to test these agents in the adjuvant setting and in patients with metastatic disease.

## **Utility of microarrays in research and treatment of gastrointestinal cancer**

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### **Introduction**

The development of microarray technology is intimately connected with the transition of molecular biology from its classical phase into its post-genomic era. Microarray technology promises not only to dramatically speed up the experimental work of molecular biologists but also to make possible a whole new experimental approach in molecular biology. Instead of investigating the complexity of biological effects by analyzing single genes of putative importance one after the other, many or even all genes of an organism can now be tested at once. First, to find out which genes are involved in a biological event and, second, to analyze in detail their interactions afterwards. Although technically still in the early stages of development, microarrays are already indispensable for two new full-genomic approaches: large-scale genotyping and gene-expression profiling. In connection with genome sequencing projects, the former gives the microarray unprecedented potential as a DNA-analytical system and the latter takes the microarray from the basic research laboratory and establishes it as a key component of high-throughput screening systems in drug research.

The January 1999 supplement of *Nature Genetics* was devoted completely to this field. More recent information is available from reviews covering general aspects, technical details, experimental solutions or biological results and specifically the field of cancer as well as from more than 200 companies worldwide engaged in the development and application of this technology. The scope of this review is therefore restricted to some examples of recent technical advances and research applications, and is focused on current trends in the movement of the microarray from being a purely research method to becoming an analytical instrument applicable in the clinic as well as in industry.

### **The present state of microarrays technology**

Working with microarrays requires the combination of at least five different components: the chip itself with its special surface; the device for producing microarrays by spotting the nucleic acids (probes) onto the chip or for their in situ synthesis; a fluidic system for hybridization to target DNA; a scanner to read the chips; and sophisticated software programs to quantify and interpret the results. Additional tools are required for extracting nucleic acids from biological

material to prepare them for the analysis. For each of these components special equipment is now commercially available. In addition, microarray components or complete systems, ready-to-use gene collections and PCR product libraries of cDNA and even comprehensive microarray studies are commercially offered as services. Usually, the different systems show very different levels of reliability and reproducibility, are not compatible with each other and require a skilled scientist to setup, commission and even to routinely run them. The value of microarray experiments still depends critically on the quality of arraying, recently made possible by bubble jet technology or maskless in situ synthesis of oligonucleotides. Microarray experiments also depend on probe and target preparation, experimental variations during hybridization and specifically on the selection of the nucleic acids affixed to the microarray surface. Further, microarray experiments depend on the homogeneity of the surface and linking chemistries on the chip as well as on background and overexposure problems during image processing.

Based on improvements in microarray surface chemistry, scanner technology and software developments, quantitative changes in transcription activity can now be measured reproducibly in the range twofold or less, except in the case of low abundant mRNAs. However, technical standards or established procedures for the exact comparison of the different technical systems or among different approaches, such as cDNA-arrays versus oligonucleotide-arrays, are still missing. Now, as before, the microarray field is moving very fast and new technical approaches and applications are emerging continuously.

A remarkable recent advance is the development of 'fluidic' microarrays, a system for massively parallel signature sequencing (MPSS). Millions of DNA-signed microbeads, each carrying a different cDNA attached by in vitro cloning, are repeatedly cycled between restriction type II cleavage, ligation steps and hybridization reactions to add decoder probes for reading the signatures. The number of microbeads carrying identical cDNAs are then counted by imaging them onto a charge-coupled device camera using a flow cell. Because ~250,000 microbeads are processed at once, even rare mRNAs can be assessed without prior knowledge of their sequence. Microbeads are also employed to attach molecular beacons that produce a fluorescence signal after binding of (unlabeled) target molecules. By encoding them with a particular dye signature, >10<sup>7</sup> randomly ordered microbeads can be analyzed simultaneously in a high-density fiber array using an imaging fluorescence system. To increase the sensitivity of

microarrays a new 'scanometric' detection system based on gold-nanoparticle-promoted silver reduction has been reported to be 100 times more sensitive than fluorescence measurement.

As a method connecting genomics and proteomics microarray technology has also been used for large-scale peptide and protein analysis. New protein microarrays can be used instead of the yeast two-hybrid system for in vitro analyzing protein–protein interactions, for identifying protein kinase substrates and for measuring interactions between proteins and low-molecular weight molecules and even low-affinity interactions. In addition, the microarray technique has been used to screen >18,000 antibodies against 15 different antigens in one experiment using high-density gridding of bacteria containing antibody genes and testing them using a solid-phase enzyme-linked immunosorbent assay (ELISA). Single-stranded nucleic acids coupled to proteins have been used to convert DNA microarrays into protein microarrays in a one-step, self-assembling hybridization process and plasma polymerized protein films have been used to fabricate DNA-arrays. Another area of noteworthy advance, and one that has long been neglected, is the proper identification of sources of noise, error analyses and quantitative treatments of systematic and stochastic errors in DNA microarray analyses.

### **Biological results from microarrays expression research**

The most widely distributed research application of DNA microarrays is gene expression profiling. It has already been proven in earlier studies to be very helpful in differentiating tumor and normal cells. Clinically significant is the demonstration that two otherwise not clearly distinguishable subtypes of non-Hodgkin's lymphomas could be differentiated by investigating 17,856 genes in specimens of patients suffering from diffuse large B-cell lymphoma. Similarly, subtypes of cutaneous melanoma were predicted based on profiling 8150 genes. Epithelial and breast cancer cells were characterized in vivo and in vitro to distinguish and classify solid tumors. The 60 cell lines from leukemia, melanoma, central nervous system, colon, renal and ovarian tissue used in the National Cancer Institute for anti-cancer drug screening revealed clearly distinguishable profiles if assayed with 9703 human cDNA probes. Fundamentally new insights have also been obtained in studies comparing highly and less metastatic melanoma cells, tumor and normal colon tissue, and acute myeloid leukemia versus acute lymphoblastic leukemia. Whether some results of this kind might be questionable has to be clarified, because aneuploidy was shown to lead to spurious correlation among expression profiles and to be more widespread than expected.

Full-genome expression profiles from 300 different mutants, physiological situations or chemical treatments of a yeast culture have been measured from 4553 genes and compared with 63 such profiles of an isogenic strain grown under standard conditions. The resulting 'compendium' database allowed the monitoring of hundreds of different cellular functions as one single assay using the microarray. This database was used to estimate that under constant conditions the level of gene induction or repression natively fluctuates in the range of twofold, but also to identify eight yeast ORFs as being involved in ergosterol biosynthesis, cell-wall structure, mitochondrial function or protein synthesis. In addition, this database allowed the discovery that the cellular target of the anesthetic drug dyclonine in humans is the neuroactive sigma factor, which shows the greatest sequence homology to the effected yeast gene *erg2p*. Using the method of singular value decomposition (SVD), the complexity of large sets of microarray expression data can be reduced to show that the 'music of genes is orchestrated' through a few simple underlying patterns.

Meanwhile, experiments including up to 15,000 genes and more have been carried out to analyze the susceptibility of murine B cell lymphoma to apoptosis after irradiation, to characterize the different gene activities between placenta and embryos in mice, to measure the response of the human intestinal cells to infection with Salmonella bacteria, and to investigate the aging brain of mice, thereby showing that results obtained with microarrays correspond quite well with the quantitative reverse transcriptase PCR, a former standard technique. Furthermore, yeast cells are shown to induce 7% of their 6035 ORFs more than fivefold after 10 minutes of exposure to low saline stress but to delay this reaction under higher stress conditions. After fractionating 'membrane-bound' or 'cytosolic' polysomes, 275 new human and 285 new yeast genes were recognized to encode membrane proteins by quantifying the mRNA from both preparations on microarrays.

Additionally, microarrays continue to be used for large-scale genotyping. Single nucleotide polymorphisms (SNPs) from over 64,000 probes have recently been analyzed with the help of one 8×8mm high-density microarray. By combining the PCR/LDR (ligase detection reaction) with microarray detection, small insertions and deletions in the BRCA1 and BRCA2 oncogenes could be detected. Large-scale screening of 2848 SNPs led to genotyping of eight mouse strains, and by analyzing 1494 human SNP loci loss of heterozygosity was demonstrated to occur during tumorigenesis.

### **Emerging applications: drug discovery**

The research efforts in expression profiling have been paralleled by applying microarray technology as component of high-throughput systems used in drug discovery and development. The application of microarrays for sequencing by hybridization (SBH) made some progress, but has not yet been established as a reliable de novo sequencing method. The re-sequencing approach, measuring the identity of an individual gene by comparing it with an already known sequence, takes advantage of the special strengths of the microarray technique for analyzing mutations, genotyping and identification of organisms, from single individual recognition up to screening of whole populations. A remarkable advance, which can be directly introduced into the practice of forensic medicine, animal or plant breeding and other applications is the development of a high-fidelity method to analyze microsatellites on electronically active DNA microarrays. Although the development of new diagnostics for routine clinical use requires a time-consuming validation process, both types of microarray applications (the gene expression profiling and the genotyping approach) presently focus on the pharmaceutical industry, clinical and point-of-care medical diagnostics. Application fields such as product quality control in the food industry, water and other environmental quality, as well as plant and animal breeding and many other opportunities for taking advantage of the potential of DNA-analysis may be ready for use even faster, if fully integrated systems become available. Such systems would no longer require the effort of a complete research laboratory.

New technical solutions to improve the currently available instrumentation and to fulfill the requirements of second-generation microarrays, such as new hybridization detection systems, new chip formats, kinetic microarrays and other systems improvements, are under development. New hybridization detection methods include the use of on-board phototransistors with traditionally fluorescent-labeled DNA, optical fiber bundles, electrochemical detection schemes that use redox-active labels and the use of conductive nanoparticles of colloidal gold and carbon nanotubes as labels. These developments are directed at improving the performance of the individual devices that form part of the five technical components of the presently available DNA microarray systems.

In addition, important innovations are also occurring that aim to replace these different components by creating a fully integrated system, one that combines all of the various modules of the present microarray technology into a single unit. Although DNA microarrays using real-

time hybridization have been reported previously, different flow-through systems have recently been developed allowing a continuous measurement of the hybridization process, either in combination with improved PCR techniques or even by integrating cell lysis and amplification into a miniaturized fluidic system. This approach extends the two dimensions of the lateral microarray resolution by the time-resolved analysis of the binding process as a third dimension. Whereas standard microarrays measure all spots at once under identical hybridization conditions, usually after several hours and after the reaction equilibrium has been reached, real-time hybridization allows the calculation of binding kinetics for every spot, not only after short times but also under different temperatures or otherwise changed hybridization conditions, resulting in an additional dimension of resolution. Flow-through systems also take full advantage of the opportunity to significantly reduce hybridization times, to re-use the microarrays and to cut their costs, which are still much too high for broad application.

Microarray systems for industrial use demand at least two types of software packages. The first is to select the oligonucleotide libraries for configuring the microarrays and for providing the reference information for each spot. This software serves to optimize the properties of the nucleotides selected for every new application according to its analytical goal. The software must be suitable to optimize the hybridization efficiency, the secondary structure of their complementary target sequences, as well as the cross-hybridization potential between the surface-bound molecules and the crude nucleic acids mixture to be analyzed. Every new genome being completely sequenced eases this task but makes the bioinformatic tools even more urgently needed for microarray technology. The second type of software has to collect and to process the primary hybridization data from each spot and to automatically extract the final analytical answer.

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## **Patogénesis molecular del cáncer de colon y recto: aplicaciones clínicas de su conocimiento**

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Los avances en el conocimiento de la biología molecular y celular del cáncer han permitido identificar alteraciones en determinados genes y sus productos que pueden ser de utilidad clínica. En algunos casos estos genes están alterados en la línea germinal siendo la base molecular de los síndromes de predisposición hereditaria al cáncer. El reciente desarrollo de nuevas tecnologías permite el análisis molecular de muestras invasivas o no invasivas recogidas de forma rutinaria. La disponibilidad de esta metodología (i.e. secuenciación semiautomática y automática, *microarrays*, PCR semicuantitativa) hace viable que, en caso de demostrar su beneficio respecto a los criterios clínicos o de laboratorio ya establecidos, se pueden introducir en la práctica clínica estos nuevos parámetros moleculares. El conocimiento de la base molecular del cáncer nos permite pasar de una medicina empírica a una medicina predictiva con aplicaciones en la estimación del riesgo de padecer un cáncer, de recidiva de un determinado tumor o de respuesta frente a un tratamiento específico.

En el presente trabajo repasaremos brevemente algunos ejemplos de cómo se está empezando aplicar en el campo del cáncer colorectal esta nueva biología predictiva: el diagnóstico molecular del Cáncer Colorectal Hereditario; la detección de mutaciones para el diagnóstico no invasivo de la enfermedad así como la introducción de nuevos factores pronósticos o de predicción de respuesta o de tolerancia al tratamiento quimioterápico.

### **Diagnóstico molecular del Cáncer Colorectal Hereditario.**

El CCR Hereditario se compone, en su mayoría, de dos grandes síndromes: la Poliposis Cólica Familiar y el Cáncer Colorectal Hereditario No Poliposis.

Poliposis Cólica Familiar. La Poliposis Cólica Familiar (*Adenomatous Poliposis Coli*) es una enfermedad hereditaria autosómica dominante responsable de menos del 1% de los cánceres colorectales. Se caracteriza por la presencia de más de 100 pólipos adenomatosos en colon y recto que, habitualmente, se inician entre los 20-30 años. El riesgo de desarrollar un cáncer a los 40 años es cercano al 100% si no se ofrece tratamiento.

La alteración molecular responsable de la PCF son mutaciones en línea germinal en el gen *APC* (*Adenomatous Poliposis Coli*), identificado hace 10 años en la zona 5q21 delimitada por deleciones presentes en pacientes con Poliposis Cólica Familiar (PCF). Se considera que más del 90% de las familias afectas de PCF contienen en la línea germinal mutaciones en el gen *APC*. Si se estudian con métodos convencionales [i.e. secuenciación directa, SSCP, DGGE o Test de la Proteína Truncada (PTT)] se deben detectar mutaciones en un 60-80%. Utilizando métodos más sofisticados como la técnica de detección de mutaciones monoalélicas este porcentaje debe aumentar hasta el 90%. La mayoría de éstas (>95%) generan una proteína truncada. Recientemente se han detectado grandes deleciones del gen hasta en un 10% de las familias.

El diagnóstico molecular directo, basado en la detección de mutaciones en el gen *APC*, es la mejor opción disponible porque ofrece ventajas respecto al diagnóstico indirecto. Entre un 25-50% de los casos identificados en un Registro son mutaciones *de novo* por lo que es la única opción; no es fácil por problemas de supervivencia y de colaboración de familiares la obtención de material de calidad de 2 pacientes afectos y de los dos progenitores necesario para realizar el diagnóstico indirecto; el tamaño de las familias es cada vez menor en nuestro entorno; la heterocigosidad de los marcadores es siempre limitada.

El diagnóstico molecular de portadores asintomáticos se debe ofrecer en la adolescencia que es el momento de inicio de las rectosigmoidoscopias en los familiares en situación de riesgo. Si se identifica la mutación responsable de la enfermedad en una familia la presencia o ausencia de mutaciones en los portadores condiciona el cribado de los familiares en situación de riesgo. Si un familiar es portador el diagnóstico molecular nos ayuda a ser más contundentes en las recomendaciones de seguimiento basadas en rectosigmoidoscopias anuales o bianuales. Si en una familia con mutación identificada, el familiar en riesgo no es portador se debe sacar del programa de cribaje avisando que tiene el riesgo de la población normal de desarrollar CRC.

Cáncer Colorectal Hereditario No Poliposis (CCHNP). El CCHNP es, probablemente, la forma más común de síndrome hereditario de cáncer colorectal (CCR) y puede representar entre el 1-6% de estos tumores. Se han adoptado criterios diagnósticos estrictos [Criterios de Amsterdam (CA) I o II], basados esencialmente en la historia personal y familiar de cáncer, con el fin de identificar estas familias. Sin embargo estos criterios pueden ser demasiado

restrictivos cuando se aplican a núcleos familiares pequeños cada vez más frecuentes en nuestro medio.

Las mutaciones germinales en los genes reparadores son una característica molecular de una proporción significativa de las familias CCHNP. Entre el 30-40% de las familias que cumplen criterios estrictos de Amsterdam presentan alteraciones en la línea germinal en los genes reparadores *hMSH2*, *hMLH1* o *hMSH6* (Weber et al, 1997); en una minoría de estas familias se han identificado mutaciones en los genes *hPMS1* y *hPMS2* o el TGF $\beta$ RII. Además se han detectado un número importante de mutaciones en familias que no cumplen los CA (Wijnen et al, 1998). La mayoría de las mutaciones patogénicas generan una proteína truncada y la consiguiente falta de función. Una proporción significativa de cambios son mutaciones que provocan un cambio de aminoácido y que, al igual que ocurre en otros síndromes hereditarios de susceptibilidad a desarrollar tumores, no siempre se conoce su potencial patogénico. En estos casos es importante disponer de pruebas de funcionalidad de la proteína y de poder analizar varios miembros afectados y no afectados de la familia para poder determinar su importancia real.

Cuando se detecta una mutación en un gen reparador ésta debe ser utilizada en el consejo genético. Recientemente se ha acordado que la presencia o ausencia en familiares en situación de riesgo de estas mutaciones en familias con mutaciones claramente patogénicas comporta cambios en el programa de seguimiento. Si no es portador, el paciente pasa a tener el riesgo de la población normal de desarrollar carcinomas y es candidato a un programa de cribado válido para la población general.

El bajo rendimiento de la detección de mutaciones de genes reparadores ha llevado a proponer el uso de marcadores menos sofisticados que nos ayuden a identificar los pacientes candidatos a estudio de línea germinal. Se ha descrito que un subgrupo de carcinomas colorrectales (7.5-28%) presentan errores múltiples de replicación del ADN - 100 veces más que en tejido normal y que en el resto de tumores - en secuencias cortas repetidas o microsatélites [(A)<sub>n</sub>, (CA)<sub>n</sub> y (CAG)<sub>n</sub>] (MSI, **MicroSatellite Instability**) (Ionov et al, 1993). En series consecutivas de CRC obtenidas en USA se ha detectado MSI en un 15-30% de tumores. En nuestro medio esta incidencia es significativamente menor (7-9%) probablemente asociada a diferencias poblacionales (González-García et al, 2000).

Existe una fuerte asociación entre MSI y CCHNP. En los estudios iniciales un 100% de los carcinomas de pacientes CCHNP eran MSI(+), tasa que disminuía al 70% si no habían mutaciones detectables o si eran adenomas (revisado en Lynch HT, de la Chapelle A, 1999). Además existe una alta heterogeneidad en la detección de MSI cuando se analizan tumores de diferentes miembros de una familia. En nuestra experiencia la incidencia de MSI en familias que cumplen CA tipo I o II –independientemente de que se detecten o no mutaciones - disminuye al 40% (González et al, datos no publicados), de acuerdo con una prevalencia de mutaciones del 30% en estas familias. Estas observaciones han llevado a proponer que la determinación de MSI en tumores se utilice para aumentar el rendimiento del análisis en línea germinal tanto en pacientes que cumplen como que no cumplen los criterios de Amsterdam. Estudios aún más recientes sugieren que la asociación de estudio de expresión de proteínas hMLH1, hMSH2 y hMSH6 puede ayudar a ser más precisos en la estrategia de análisis molecular en línea germinal.

### **La utilidad de los marcadores moleculares en el diagnóstico no invasivo del cáncer colorectal**

El desarrollo de la neoplasia es el resultado de la acumulación de alteraciones genéticas en oncogenes y genes supresores. En los últimos años se ha identificado de forma bastante precisa la base molecular de la secuencia adenoma carcinoma colorectal. Las mutaciones en el gen *K-ras* (incidencia 40%) ocurre de forma preferente durante el desarrollo del adenoma mientras que las mutaciones en el gen supresor *p53* (incidencia 50-70%) se asocian a la transición adenoma-carcinoma. Hoy en día es posible detectar la presencia de alteraciones genéticas en el DNA extraído de las células exfoliadas en heces gracias al desarrollo de técnicas de alta sensibilidad que permiten detectar un alelo mutado en medio de una mayoría de células normales. Se detectan mutaciones en el gen *K-ras* en heces en más del 90% de carcinomas que la contienen mientras que la utilidad del diagnóstico molecular no invasivo disminuye en los adenomas. La combinación de detección de alteraciones en *p53* y en el marcador de MSI BAT26 no aumenta la especificidad para el diagnóstico de cáncer. Ya se está evaluando la utilidad de la detección de alteraciones genéticas en heces en pacientes con riesgo elevado de desarrollar cáncer con el fin de evaluar su posible utilidad como biomarcador de riesgo.

## **La evaluación de nuevos factores pronósticos en cáncer colorectal**

En cáncer colorectal la presencia de invasión de la pared vascular y, sobre todo, la presencia de metástasis ganglionares son factores pronósticos potentes reflejados en las clasificaciones TNM y Dukes. A pesar de ello todavía existe una proporción significativa de tumores cuyo estadio no es acorde con su evolución clínica. La detección de alteraciones en oncogenes (i.e *K-ras* en el 40%) y genes supresores (mutaciones en el gen *p53* en el 50%, silenciamiento del *p16* en el 50%, pérdida alélica en 18q21 en el 50-70%) en una alta proporción de estos tumores ha llevado a evaluar su posible utilidad pronóstica. El reto en este campo está en demostrar que el uso de marcadores moleculares ofrece mejor información que las clasificaciones clínico-patológicas aceptadas. En estudios prospectivos se ha podido demostrar que las mutaciones en el gen *K-ras* y/o *p53* se asocian a una mayor agresividad del tumor. Sin embargo cuando se consideran sólo los casos intervenidos con intención radical no ofrece información adicional al estadio tumoral. Al añadir la información de silenciamiento de los genes supresores *p14* y *p16* se ha podido evidenciar que la presencia la alteración en los genes *p16* y/o *K-ras* son un factor pronóstico independiente en análisis uni y multivariante. Estas diferencias se mantienen en casos sometidos a resección radical lo que sugiere que el análisis combinado de *K-ras* y *p16* puede ser de utilidad pronóstica en el cáncer colorectal humano. La pérdida alélica en 18q21 también parece ser un buen marcador pronóstico en cáncer colorectal humano a pesar de que no se ha podido confirmar que su poder discriminador se asocia a pérdida de expresión de la proteína DCC. Como alternativa a la detección de alteraciones genéticas específicas existe la posibilidad de cuantificar el daño genómico o la desregulación génica. La determinación del contenido de DNA mediante citometría de flujo (DNA index) o bien de la tasa de desequilibrios alélicos o la desregulación génica global se asocian a mal pronóstico si bien su utilidad pronóstica como factor independiente del estadio no ha sido establecida.

## **Predicción de respuesta al tratamiento**

El tratamiento quimioterápico es efectivo en una proporción de los pacientes que lo reciben y asocia, con frecuencia, efectos secundarios. Si bien el tratamiento se selecciona en función de las características del tumor y del paciente no ha sido hasta ahora posible predecir quien responderá y quien no. El estudio de un gran número de genes que afectan la actividad de la droga y su metabolismo (farmacogenómica) ofrece la posibilidad de ofrecer un tratamiento a la medida. Por ejemplo, variantes de la Dihidropirimidina dehidrogenasa que

afectan la actividad del enzima, pueden también influir sobre el metabolismo y, por consiguiente, la neurotoxicidad del fluorouracilo. Adicionalmente las variantes del gen Timidilato Sintasa asociadas a menor actividad se asocian a una mejor supervivencia en pacientes tratados con 5-FU. En cuanto al tumor se conoce que los tumores MSI son parcialmente resistentes a la terapia con 5-FU y que existen datos controvertidos sobre la posible implicación de p53 en resistencia al tratamiento. Estos y otros aspectos de la farmacogenómica del cáncer colorectal serán desarrollados en mayor detalle por otros ponentes de este congreso.

## **Conclusión**

Existen nuevos parámetros moleculares que empiezan a ser introducidos en la práctica clínica que ayudan a predecir tanto el riesgo de desarrollar un tumor como el comportamiento biológico o la respuesta al tratamiento del mismo. En los próximos años será necesario evaluar, en contextos clínicos controlados, su utilidad real e incorporarlos a la rutina clínica con la objetivo de mejorar la esperanza de vida de los pacientes oncológicos.

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## **The adjuvant treatment of colon cancer**

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Colorectal cancer is most common in economically developed countries, particularly in parts of Europe, North America and Australia. The annual incidence varies between 55 and 0.7 per 100,000 people, respectively, in high- and low-risk populations. Colorectal cancer is one of the leading causes of cancer-related deaths in the Western world. Every year, colorectal cancer is responsible for an estimated 400,000 deaths worldwide. Approximately 60,000 people die from colorectal adenocarcinoma among the 150,000 new cases which are diagnosed in Europe each year.

Most patients (70%) who have colorectal cancer present with apparently localized disease. The remaining 30% have advanced disease at diagnosis, 25% of whom have distant metastatic disease and 5%, locally advanced disease. One quarter of colorectal cancer cases are confined to the rectum, while the remainder are located in some part of the colon.

Surgery is the only curative option for patients with colorectal cancer. Colorectal cancer is not uniformly fatal, although there are large differences in survival depending on stage of disease. Pathologic stage is presently the most important determinant of prognosis. The classification system described by Dukes in 1930 is still widely used. However, the original Dukes' system no longer fulfills the requirements of modern tumor staging, as it fails to take into account distant metastases, the number of lymph nodes involved, and carcinomas limited to the submucosa. Therefore the TNM classification of the American Joint Committee on Cancer (AJCC) is currently recommended for daily use and in clinical trials. The prognosis depends on the stage at which the tumor is diagnosed. In patients with a stage I tumor (pT1 or pT2N0M0), the 5-year survival amounts to > 90%. In patients with a stage II tumor (pT3 or pT4N0M0), the survival is variable. In patients with a pT3N0M0 tumor, the 5-year survival amounts to approximately 70%, while in patients with a pT4N0M0 tumor, the 5-year survival is much lower and is only around 30%. In patients with stage III tumors (pTXN+M0), the 5-year survival is 30-50%. In patients with metastatic colorectal cancer (stage IV), the 5-year survival is < 5% (1,2).

Although the TNM classification is generally used to determine therapy for a given patient, other factors are thought to have an independent influence on outcome. Risk of recurrence appears to increase with bowel perforation, obstruction, the presence of venous, perineural or lymphatic invasion, poorly differentiation and elevated CEA level. More recently, it has been

suggested that other factors might influence the recurrence rate and the prognosis of patients with resectable colon cancer, e.g.: DNA aneuploidy, the detection of micrometastases in lymphnodes by PCR, the thymidylate synthase level, overexpression of the p53 gene, alterations in chromosome 18q (deleted in colon cancer (DCC) suppressor gene), and microsatellite instability (MSI) (3-8). Although these factors and other new molecular factors can become extremely important in the future in the management of patients with colon cancer, neither of these factors are actually to be used systematically outside a clinical trial.

The adjuvant therapeutic modalities of colon and rectal cancer have to be differentiated. Rectal cancer can be defined as any tumor that is located either partly or entirely below the peritoneal reflection. This location makes it more difficult for surgeons to obtain wide margins at resection and is associated with a higher incidence of locoregional failure. Adjuvant therapies in patients with high-risk rectal cancer (stages II and III) must include an adequate surgical approach (Total Mesorectal Excision=TME) usually combined with a post- or preoperative radiotherapy, probably in combination with chemotherapy, to reduce the risk of local failure and of distant metastases and ultimately also to influence overall survival (7).

Adequate surgery by experienced surgeons is the basis of the treatment of resectable colon cancer and determines the prognosis. Surgery is the only curative option for patients with colorectal cancer. It has also been shown that the prognosis is also correlated with the number of detected and removed lymph nodes. An adjuvant therapy after surgery is administered with the intent to target residual occult viable cells and eradicate them before they become established and relatively refractory to intervention. Important considerations are the risk-benefit ratio of such treatment and the need to achieve a balance between maximum chance of cure/prolonged survival and tolerance or side effects.

### **Stage III colon cancer**

The results of the adjuvant treatment in colon cancer are considered as one of the most important advances in clinical oncology over the last decade because of its impact and the implications of life savings, also due to frequency of colon cancer (1,2).

The intergroup trial reported in 1990 by Moertel et al. (INT-0035) was the first large-scale study to demonstrate a significant survival benefit and a reduction in recurrence risk after postoperative treatment in patients with resected stage III colon cancer. This trial randomized 1,296 patients to one of three arms: (1) surgery alone, (2) surgery plus 12 months of

levamisole, or (3) surgery plus 12 months of 5-fluorouracil (5-FU) plus levamisole. The study showed a 15% absolute reduction or  $\pm$  40% relative reduction in risk of recurrence and a 33% relative reduction in the overall death rate with the combination of surgery plus 5-FU/levamisole. Levamisole (LEV), a phenylimidothiazole compound, is an antihelminthic agent with immunomodulatory activity (T-cell stimulation), that has no effect on survival used alone adjuvantly (9,10).

Since the combination of 5-FU and leucovorin (LV) has proved to be superior to 5-FU alone in patients with advanced colorectal cancer, a number of studies have confirmed the efficacy of 5-FU modulated by LV as adjuvant treatment, when compared with a no treatment control arm (11). The NSABP (National Surgical Adjuvant Breast and Bowel project) protocol C-03 indicated a disease free (73% versus 64%) and overall (84% versus 77%) survival advantage for the 5-FU/LV combination when compared with MOF (methyl-CCNU, oncovin, 5-FU) at 3 years for patients with Dukes' stage B and C colon cancer (12). The control arm in this study (MOF) had previously shown a survival advantage in the adjuvant setting. The Canadian and European consortium trial (IMPACT) compared adjuvant treatment with high-dose 5-FU and LV with no treatment in nearly 1500 patients; they demonstrated a 22% reduction in mortality at 3 years, both in Dukes' B and C patients (13). A similar in design but smaller Italian study (Givio) showed a 39% reduction in mortality for the same group of patients (14). With a median follow-up duration at 72 months, an Intergroup study indicated that patients who received a combination of 5-FU and low dose LV over 6 months experienced significant improvement in time to relapse ( $P < 0.01$ ) and survival ( $P = 0.02$ ) compared with control patients treated with surgery alone. Based on indirect comparisons of the trials with 5-FU/levamisole and 5-FU/leucovorin it was suggested therefore that both regimens were equally effective.

More recently the results of 3 large adjuvant American trials have been presented in which several thousands of patients have been treated. In a large, randomized study by the North Central Cancer Treatment Group (NCCTG) and the National Cancer Institute of Canada (NCIC), it was shown that there is no benefit associated with administration of a full year of chemotherapy compared with just 6 months of treatment with the same regimen (15). In the same study, it is shown that, if only 6 months of chemotherapy is administered, patient survival was significantly inferior with the 5-FU plus levamisole regimen compared with the 3-drug 5-FU plus levamisole plus leucovorin regimen (15). At the 1998 ASCO meeting, the Intergroup reported moreover that there is no additional benefit from the addition of levamisole when 5-FU/leucovorin is given and that 6 months of treatment with 5-FU/leucovorin is as efficient as

12 months of 5-FU/levamisole (INT-0089) (16). The NSABP C-04 study showed similar results (Table 1) (17). Taking into account also the increased toxicity of the 3-drug combination (5-FU/leucovorin/levamisole) compared with the combination of 5-FU/leucovorin, it is accepted that a treatment with 5-FU/leucovorin for 6 months is nowadays the standard treatment in Dukes' C colon carcinoma (18,19).

Two regimens of 5-FU/LV are proposed in the USA: weekly LV (500 mg/m<sup>2</sup>) plus 5-FU (500 mg/m<sup>2</sup>) during 6 weeks followed by 2 weeks of rest or the "Mayo Clinic Regimen": LV (20 mg/m<sup>2</sup>) + 5-FU (425 mg/m<sup>2</sup>) d. 1-5 repeated every 4 weeks. In Europe the Mayo Clinic Regimen is usually proposed as the standard regimen. Several new ongoing studies are evaluating the role of other 5-FU/LV regimens in the adjuvant treatment: infusional 5-FU, infusional 5-FU/LV and shorter duration (3 months). It has indeed been shown that infusional 5-FU/LV regimens are more efficient in terms of response rate and time to tumour progression (TTP) than bolus 5-FU/LV regimens in patients with advanced colorectal cancer (20). Despite greater technical requirements the tolerance of infusional 5-FU regimens was better than of bolus regimens in patients with advanced colorectal cancer. Preliminary results of 2 studies have shown the feasibility of infusional regimens and a different toxicity pattern compared to bolus regimens in the adjuvant treatment of colon cancer. These preliminary results showed an identical disease free survival and median survival (21,22).

### **Stage II colon cancer**

The question whether stage II or Dukes' B2 cancer patients should be treated with an adjuvant chemotherapy remains controversial in 2000. Most of the published trials on the adjuvant treatment of colon cancer include as well stage II as stage III colon cancer. Data from large prospective trials in stage II colon cancer alone that allow definitively to draw conclusions on the role of adjuvant treatment in this setting are still lacking. The INT-0035 trial, which compared 5-FU plus levamisole with surgery alone showed a similar reduction in the rate of recurrence (32%) in stage II as was observed in stage III cancer. No benefit in overall survival was shown, probably because of the relative lack of power of this study (9,10).

The International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) investigators, after combining five separate trials in which patients were randomized to postoperative fluorouracil and folinic acid (FU + LV) or to no further therapy, conclude that their analysis does not support the routine use of FU + LV in all patients with B2 colon

cancer. The 5-year overall survival was 80% and 82% and the event free survival 73% and 76% resp. for controls and for 5-FU/leucovorin treated patients (23).

However, the National Surgical Adjuvant Breast and Bowel Project (NSABP) group, after combining data from four of the group's trials including stage B and C colon cancer patients, reports that patients with Dukes' B colon cancer benefit from adjuvant chemotherapy. The relative reduction in mortality, recurrence or disease-free survival event was in most instances of the NSABP trials as great or greater for Dukes' B patients than for Dukes' C patients. The mortality reduction was 30% for Dukes' B patients and this occurred irrespective of the presence of absence of adverse prognostic factors (24).

Therefore probably only a subgroup of stage II patients really benefits from postoperative treatment (18,19,25,26). The value of molecular prognostic factors such as aneuploidy, expression of p53 or p21, microsatellite instability (MSI), overexpression of thymidylate synthase, and absence of expression of the deleted-in-colorectal cancer gene (DCC) are actually under investigation for stratifying patients for an adjuvant treatment. Clinical and pathologic prognostic factors such as young age, perforation or occlusion as presenting symptom, and the presence of perineural, venous or lymphatic invasion can actually be used to identify patients at higher risk for recurrence and to select stage II patients for an adjuvant treatment outside of a clinical trial.

### **Portal vein infusion**

Several randomised trials have been performed to study the effect of an intraportal infusion of 5-FU, administered immediately after the operation. The rationale of this treatment was that colorectal cancer recurrences are often seen in the liver. Initially a number of positive results have been reported. These could, however, not always be confirmed. A meta-analysis of 9 trials of adjuvant postoperative intraportal chemotherapy was performed. A small but significant benefit in survival was found: the reduction in death was 13% (27). It can however, not be explained why the incidence of liver metastases was not lower. This suggests a systemic effect of intraportal chemotherapy that could be possibly attributed to the early postoperative administration of chemotherapy. In contrast, a more recent well performed large randomised EORTC trial could not show an effect of intraportal adjuvant chemotherapy (28). We can therefore conclude that intraportal chemotherapy is an interesting concept, but that the systematic use in clinical practice should not be advised.

Another interesting technique currently under investigation in the adjuvant treatment of colorectal cancer is intraperitoneal chemotherapy, in combination with systemic treatment. Such an approach would have the potential advantage of acting on both microscopic peritoneal tumour spread as well as on liver micrometastases, since intraperitoneal 5-FU is absorbed through the portal vein and results in a high 5-FU concentration in the portal blood. Although the benefit of such an approach is not proven, a small randomised study from investigators in Austria indicates a possible advantage over conventional adjuvant therapy. Patients with resected Dukes' B or C colon cancer were assigned to either intravenous and intraperitoneal 5-FU/LV or intravenous 5-FU/LEV as the "standard therapy" arm. A preliminary analysis after a median follow-up time of 27 months showed a significant disease free survival advantage in favour of the experimental arm (17/94 versus 35/96 recurrences,  $P=0.0015$ ) (29).

The EORTC performed a 4-arm trial to study the role of postoperative regional chemotherapy in association with systemic chemotherapy (6 months 5-FU/leucovorin versus 5-FU/levamisole). Results are not yet available.

### **Immunotherapy**

A growing interest in the immunological treatments of malignancy has led to the development of both specific and non-specific immunotherapy adjuvant trials in colorectal cancer. One of the first studies (NSABP C-01) had a three arm randomisation to either no postoperative therapy, MOF chemotherapy, or intradermal BCG. At 5 years' follow-up, the BCG treatment group demonstrated a slight survival advantage compared with surgery alone, although there was no difference in terms of disease free survival in the two groups (30).

The concept of using vaccines to induce specific immunity against carcinomas has been actively pursued over the last 2 decades. Early attempts to induce tumour regression in cancer patients by inducing tumour-specific immunity with autologous or allogeneic tumour cell vaccines were not successful. A European trial showed a longer recurrence-free period and a risk reduction for recurrence but no survival advantage for active specific immunotherapy (ASI) with an autologous tumour cell-BCG vaccine in the adjuvant treatment of stage II colon cancer (31). An American trial, however, did not show a clinical benefit in patients with stage II and III colon cancer (32).

Another direction of immunotherapy is the development of monoclonal antibodies. The murine monoclonal antibody against the 17-1A antigen, Panorex, has been shown in a small

randomized study to reduce the mortality with 32% and the relapse rate with 23% at a median follow-up of 7 years in Dukes' C colorectal cancer (33). This monoclonal antibody plus 5-FU/LV is now being compared with 5-FU/LV alone in 2 large scale randomized phase 3 studies in stage III colon cancer. The European trial has been reported. The survival and disease free survival of the combination of 5-FU/LV + Panorex were identical than the survival and disease free survival of 5-FU/LV. In stage II colon cancer a randomized trial of this monoclonal antibody after surgery versus surgery alone is ongoing (CALGB-EORTC).

### **New drugs**

The role of the oral fluoropyrimidines, capecitabine and UFT (uracil and tegafur), in the adjuvant treatment of colon cancer is actually under investigation in large scale randomized phase III studies.

The topoisomerase I inhibitor irinotecan (CPT-11) and the diaminocyclohexane platinum derivative, oxaliplatin, are 2 new active drugs in advanced colon cancer and hold promise as potentially effective drugs in early colon cancer. Large phase III trials are at present ongoing to evaluate the role of irinotecan in association with 5-FU/LV (Petacc 3 and 4 trials) and of oxaliplatin also in combination with 5-FU/LV in the adjuvant treatment of stage II and III colon cancer.

Table 1.

Trial	Regimen	N patients	5-Year Disease-Free Survival Rate (%)	5-Year Overall Survival Rate (%)
NCCTG-NCIC (Ref. 15)	5-FU/levamisole (6 mo)	230	58	60
	5-FU/folinic acid (Mayo Clinic regimen) + levamisole (6 mo)	225	63	70
	5-FU/levamisole (1 yr)	228	63	68
	5-FU/folinic acid (Mayo Clinic regimen) + levamisole (1 yr)	232	57	63
INT 0089 (Ref. 16)	5-FU/levamisole (1 yr)	833	56	63
	5-FU/folinic acid weekly (8 mo)	946	59	65
	5-FU/folinic acid (Mayo Clinic regimen) (6 mo)	953	60	66
	5-FU/folinic acid (Mayo Clinic regimen) + levamisole (6 mo)	827	60	67
NSABP C-04 (Ref. 17)	5-FU/levamisole (1 yr)	691	60	70
	5-FU/folinic acid weekly (1 yr)	691	65	74
	5-FU/folinic acid weekly + levamisole (1 yr)	696	64	73

5-FU = Fluorouracil; NCCTG = North Central Cancer Treatment Group; NCIC = National Cancer Institute of Canada; INT = Intergroup; NSABP = National Surgical Adjuvant Breast and Bowel Project

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## **Controversies in the treatment of advanced colorectal cancer**

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One controversial issue remains the optimal mode of administration of 5-FU/LV. Bolus regimens is still widely applied but will probably soon be outdated. An important reason is that combining bolus regimens with CPT-11 or oxaliplatin is too toxic [1,2]. Furthermore it is apparent that infusional 5-FU/LV on itself has a superior therapeutic index[3], while it can be combined safely with CPT-11[4] and with oxaliplatin[5]. 5-FU/LV + CPT-11 has been approved by the FDA, based on two trials that demonstrated a survival benefit for 5-FU/LV + CPT-11 over 5-FU/LV alone [6,7] and is also approved in Europe, while 5-FU/LV + oxaliplatin is approved in Europe. Some now consider 5-FU/LV + CPT-11 as current standard treatment but it remains controversial whether all patients should receive this combination up front or that a sequential approach (5-FU/LV first and upon failure in suitable patients the addition of CPT-11 or oxaliplatin to 5-FU/LV) results in an equal benefit.

Some oral 5-FU prodrugs have been approved for first line treatment in the US and Europe but it remains to be seen whether they will in part replace infusional 5-FU/LV.

New “mechanism-based” drugs (Iressa, cetuximab etc.) are now coming into clinical trials and may possibly be used in combination with chemotherapy to improve therapeutic results.

Lastly an important issue is whether it will be possible to determine the means by which physicians can individually tailor new therapies, i.e. select new agents, either as monotherapy or in combination, as best treatment on the basis of individual patients’ tumor biological markers.

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## **Novel therapies for colorectal cancer**

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The past decade has seen the clinical introduction of two effective cytotoxic drugs for colorectal cancer. What will the next decade bring? There is significant pre-clinical and early phase trial research assessing the importance of a range of signal transduction inhibitors (ras, EGE receptor, tyrosine kinase, protein kinase C isoforms, MAP kinase, etc) which may be effective as single agents but hold the promise of synergistic interactions with conventional cytotoxic drugs. There has also been an up-surge in interest in immunotherapy, especially for early stages disease, and although recent trial results of monoclonal antibody therapy have proved disappointing, T-cell based vaccination strategies, either adoptive transfer or immunisation against tumour associated antigens like CEA, are entering a range of clinical trials. Gene therapy has taken longer to develop than initially hoped, but there are gene strategies in phase I clinical trial which are certainly showing enough interest to continue into later stage trials. Vascular biologists have identified a range of endothelial targets which have led to a number of new therapeutic inhibitors entering the clinic, both as single agent and in combination with conventional cytotoxics. So, the next decade is set to be equally as interesting as the last as oncologists will be faced with increasing treatment options for colorectal cancer.

## **Adjuvant treatments for gastric cancer after R0 resection**

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Even if gastric cancer incidence is decreasing since more than 20 years (1) its prognosis remains poor (2, 3) and no significant amelioration has been achieved by using adjuvant chemotherapy with an overall survival around 15% (4). These last ten years most of the discussion has focussed on the quality of the surgical resection which has to be microscopically complete (R0 resection) and to remove a sufficient number of lymph nodes with the recommendation of performing D2 resection rather than D1 resection even if no randomised study has clearly demonstrated a survival advantage (5) in part related to an overmortality related to splenectomy and distal pancreatectomy, however some reports have demonstrated that the number of LN removed was a significant prognostic factor (6) and that a modified D2 resection without splenopancreatectomy was a reasonable recommendation.

Even if the chemotherapy for metastatic gastric cancer (GC) has made some progress these last 20 years the efficacy of adjuvant chemotherapy is still under discussion.

### **SYSTEMIC CHEMOTHERAPY**

#### **Adjuvant monotherapies**

In studies conducted more than 30 years ago fluoropyrimidines (5-fluorouracil or 5-FU) did not demonstrate any efficacy (4) and oral doxifluridine (5'-DFUR) was not superior to 5-FU in a phase III trial conducted in 485 patients with stage II or III GC (5). Mitomycin C (MMC) has given some positive results in one Spanish trial on 134 patients versus surgery alone with a 10 year survival of 39% vs 26% significantly superior to the surgery alone arm (6) ; however this study has a low power because of the small number of patients included and has not changed our policy. It has even been excluded of one meta-analysis (7) and is not a standard in Europe

#### **Adjuvant polychemotherapies**

Results from trials using polychemotherapies are discordant !

The 5-FU-methyl-CCNU combination given for 2 years gave in one study from the GITSG a 5 year survival advantage of 15 p. 100 in favor of the treated group (47 vs 33 p. 100,  $p = 0,03$ ) (8) ; however the analysis was not done on intent to treat and other studies, using the same schedule, failed to reproduce these positive results. This fact and the toxicity of nitrosourea,

especially the risk of secondary leukemia and myelodysplasia, explain that this combination has never been a standard (9).

The second generation protocols, in particular the FAM protocol (5FU + adriamycine + mitomycine C) which has been a standard in the eighties, has been evaluated in 3 studies in adjuvant without benefit in favor of the treated group (10-12). However in the Coombes et al study there was a borderline survival advantage for the subgroup of patients with infiltrative tumours, but the 2 other studies were completely negative (10).

The third generation protocols incorporated the cisplatinum (CDDP). We have recently reported a trial using the classical monthly 5FU-CDDP combination (FUP protocol) for 4 cycles after an immediate post-operative administration of 5 days of continuous iv 5FU alone ; 260 patients have been randomized and the 5 year survival rate was 43% for the control arm and 48% for the chemotherapy arm and the difference was not significant (13). This study demonstrated that the FUP protocol has no major activity in adjuvant and that the Douglass'hypothesis that early iv chemotherapy may be an explanation for a difference in the results from Japanese compared to occidental trials seems not confirmed (14). Another study from Italy, on 274 patients, using a combination of etoposide, adriamycine, CDDP (EAP), also failed to demonstrate a significant benefit in favor of the chemotherapy (5 year survival rate was 48% for the control arm and 52% for the chemotherapy arm ; ns) (15).

The possible role of oral chemotherapies has been also investigated : In Japan, a combination of MMC and 5FU twice a week for 3 weeks followed by 18 months of oral chemotherapy (UFT) has been tested on 579 patients in a japanese study (188 T1, 323 T2), and in this « good risk » group of patients there was no survival difference at 5 years (85.8% in the control group versus 82.9% in the chemotherapy group) (16). In Spain the association MMC + UFT has been tested after the positive results reported with the MMC (6) ; the same team has reported a trial which compared the MMC - UFT combination versus mitomycine C alone and after inclusion of only 85 patients and a median follow-up of 62 months they reported a 5 year survival of 67% for the combined chemotherapy versus 44% for the mitomycine C group ( $p = 0,04$ ) (17). Another study has tested this UFT-mitomycine C combination versus surgery alone in 148 patients and reported a borderline difference in the 5 year survival in favor of the UFT-MMC group : 56% vs 51% for the control group and a superior progression free survival (18). These 2 studies are in favor of a possible role for oral chemotherapies and were tolerated with no grade 4 toxicity and only one grade 3 toxicity reported in the last study (18).

## **Meta-analysis**

The first meta-analysis was done on randomized trials with a control group treated by surgery alone and reported between 1980 and 1991. This was not a meta-analysis done on individual data and the data were not updated. The relative risk of death was reduced at 0.88 in the adjuvant chemotherapy group but this decrease was not statistically significant (7). This meta-analysis has been criticized for its lack of power and its trials' selection which was disputable (19). Later these authors reported an actualisation of this meta-analysis suggesting a borderline benefit in favor of the chemotherapy. A second meta-analysis in 1999, was done on 13 randomized trials excluding the studies testing any kind of immunotherapy alone or combined to chemotherapy or including patients with residual disease (R1 or R2 resection) ; with 1990 patients this analysis demonstrated a 20% reduction in the risk of death for the chemotherapy group (0.80 ; range : 0.66 – 0.97) (20). A third one has been reported in 2000 ; on 3658 patients from 20 studies, but without actualised and individual data, this study confirmed a 18% reduction in the risk of death for the chemotherapy group [0,75 – 0,89] (21). A fourth meta-analysis, reported only in abstract form and performed on occidental trials reported between 1981 and 1999 and selected on medline, reported on 2913 patients a similar risk of death reduction in favor of the chemotherapy group (RR death = 0.86 ; range 0,74 – 0,99) but, once again, without individual data (22). A fifth meta-analysis was conducted in Sweden which was an update of the previous study from Hermans (7) , this meta-analysis emphasized 21 studies, concerns 3962 patients, and reports also a significant benefit in favor of the chemotherapy with a hazard ratio (HR) of 0.84 (95% confidence interval : 0.74-0.96) ; however when they split this study into trials from Asia and from western countries they observed a very significant HR of 0.58 for asiatic trials in favor of chemotherapy but no benefit for western trials (HR : 0.98) (54) without any explanations for this difference even in the type of surgery and amount of potential residual microscopic disease assessed on the quality of lymph nodes dissection..Even if these meta-analysis are in favor of a significant but marginal activity of the adjuvant chemotherapy they cannot be considered as a proof because the statistical analysis were not done on individual and updated data.

## TARGETED AND LOCAL THERAPY

### **Radiotherapy (RT) and radio-chemotherapy (RT-CT)**

Trials on postoperative RT failed to demonstrate any benefit on survival, even if locoregional recurrence are frequent after resection of gastric cancer, especially in case of T4 or N positive tumors (34).

Concerning RT-CT associations, one intergroup US trial has been recently published and reported positive results on survival. This trial has randomised 556 patients after resection of gastric or gastroesophageal junction adenocarcinoma between surgery followed by a combination of CT and RT-CT or surgery alone. The adjuvant treatment consist of 5 days of 5FU bolus (425 mg/m<sup>2</sup>) plus 20 mg/m<sup>2</sup> folinic acid (FA) followed by a RT-CT (45 Gy, in 25 fractions and within 5 weeks potentialized by systemic 5FU-FA administered the first 4 and the last 3 days of RT) and followed by 2 cycles of 5 days of 5FU bolus plus FA. There was a significant advantage in the overall survival for the adjuvant RT-CT group with a median of 36 months compared to 27 months and a significant worst risk of death for the surgery alone arm (HR = 1.35 ; p=0.005). There was also a significant increase in the relapse free survival (30 vs 19 months). However the value of this trial has been discussed because only 10% and 36% of the patients got a D2 or D1 dissection from their lymph nodes and 54% had a D0 dissection which is less than a complete dissection of the N1 nodes! There were also many toxicities and especially grade 3-4 hematological toxicity in 54%, digestive in 33% and infectious in 16% with 3 toxic death and only 64% of the patients who received the complete protocol treatment.

There is also some discussion on the 40% survival at 3 years and 25% at 5 years for the control group because this seems inferior to those reported in European trials (35). One may also ask the question of the curative role of the RT-CT in patients with insufficient surgery. Presently there is a discussion on the future place of RT-CT as a standard adjuvant treatment after resection of advanced gastric cancer, but in many ongoing protocol it will be considered as a suitable control arm as well as a recommended treatment in case of D0 dissection or R1 resection. New protocols are needed to optimized the schedule of RT-CT.

### **Locoregional chemotherapies**

Intraperitoneal chemotherapy (IPC) is a logical approach if we consider the frequency of peritoneal carcinomatosis and the pharmacokinetic properties of intraperitoneal administration of antineoplastic agents which induce, for most of them, an increased portal concentration (23). One study in a small group of patients testing 5 cycles of IPC using a 5FU-CDDP

combination reported in an uncontrolled trial the feasibility of this technic and a rather good survival (24). One Japanese phase III trial on 141 patients has reported a decreased rate of peritoneal recurrences after IPC plus hyperthermy after surgery compared to surgery alone and an increased survival at 8 years (62% versus 49%) (25); these results support the possible role of adjuvant IPC however this treatment may be toxic and no other trial has confirmed these results.

### **Immunotherapy**

This approach has been quite exclusively tested by Japanese and more than 20 trials have been so far reported. Most have tested the combination of CT and immunotherapy and used non-specific immunostimulants as bacterial extracts from *Streptococcus Pyogenes* (OK-432 par exemple), *Schizophyllum*, *Nocardia rubra*, *Streptomyces olivoreticuli*, mycotic extracts like polysaccharide-K (PSK) polysaccharidic protein from *Coriolus versicolor* and chemical product like levamisole, cimetidine or poly-adenylique poly-uridilique acid (polyA-polyU).

Most failed to demonstrate a survival advantage however in some studies subgroup of patients seemed to have a better outcome compared to chemotherapy alone. This was the case of a small trial reported by Koyama et al for T3, T4 tumor when *Nocardia Rubra* was added to tegafur (26) and another small trial for patients with T2, T3 tumor receiving picibanil (OK-432) was added to chemotherapy using 5FU, mitomycine C, cytarabine and ftorafur compared to chemotherapy alone and a control group receiving no treatment after surgery (27).

The results of larger trials have been controversial. One large multicenter trial on 5484 patients from 266 centers which has compared a classical chemotherapy in Japan (Mitomycine C + Ftorafur) to this chemotherapy combined to OK-432 or PSK or a combination of PSK and OK-432 has been negative but 30% of the patients were not included in the final analysis (28). Some other trials are less negative; the first has been reported by the Kyoto Research Group Digestive Orient Surgery (1011 patients randomised and 970 analysed) comparing a group receiving a combined chemotherapy (mitomycine C + 5FU and Ftorafur) to a group receiving this chemotherapy plus oral OK-432 or subcutaneous OK-432 which demonstrated a small but significant survival benefit in favor of the OK-432 groups ( $p < 0,05$ ) (29). The other trials using PSK or OK-432 have also suggested some benefit in favor of the immunotherapy arms. These results prevent to conclude on the efficacy of bacterial extract combined to adjuvant chemotherapy in resected gastric cancer but underline the necessity to conduct trials in western countries when PSK or OK-432 will be available.

Polyadenylique-polyuridilique acid (polyA-polyU) has been tested in Corea combined to chemotherapy (5-FU + adriamycine) versus the same chemotherapy in 224 randomised patients which demonstrated a survival advantage in favor of the poly-A poly-U arm, especially for N1 patients (30).

The levamisole has shown no efficacy in a limited trial (31) and the cimetidine (400mg per os bid) which seemed favorable in a small trial conducted in an heterogeneous group of patients (including stage IV patients!) (32) was not effective in a larger trial conducted in 442 patients vs placebo (median survival : 13 months vs 11 months ; ns) (33).

### **Conclusion**

There have been few progress in adjuvant treatments for resected gastric cancers. Polychemotherapies are poorly effective apart the UFT + mitomycine C combination which warrants confirmatory trials and the ECF combination which is presently tested in a large randomised trial in UK (MAGIC trial). Immuno-chemotherapy combination using PSK or OK-432 are probably interesting but they are not available in Europe. The most striking progress is the combination of chemo-radiotherapy which, especially in case of insufficient surgery (less than D1 dissection) significantly improve the overall survival and will probably be the control arm of many future trials, however better and less toxic chemo-radiotherapy protocols warrant to be developed and tested.

The place of intraperitoneal chemotherapy, with or without hyperthermia also warrants to be further explored as in the french trial from the "Association de Recherche en Chirurgie", especially in patients with linitis plastica which have a frequent peritoneal dissemination.

Table 1. Adjuvant Polychemotherapy resected gastric cancers (trials excluded from the Earle and Maroun meta-analysis (20) are not reported).

Réf	Protocole	Number of patients	Survival (5 years)	Commentaries
(36)	Surgery alone	26	NS	Small study
	5FU + VLB + CPM	27		
(37)	Surgery alone	71	44%	p < 0.03 ; methyl-CCNU is toxic and no more used
	5FU + MeCCNU	71	59%	
(38)	Surgery alone	54	~ 50%	NS -
	5FU + BCNU	49	~ 50% (S. 4 years)	
(39)	Surgery alone	68	38%	NS;methyl-CCNU is toxic and no more used
	5FU + MeCCNU	66	39% (S. 4 years)	
(40)	Surgery alone	89	44%	NS
	5FU + MeCCNU	91	47% (S. 4 years)	
(41)	Surgery alone	69	50%	NS
	5FU + MeCCNU	75	50%	
	5FU + MeCCNU + lev	69	50%	
(10)	Surgery alone	133	39%	NS ;but possible benefit for T3-T4 (p = 0.21)
	5FU + ADR + MMC	148	44%	
(42)	Surgery alone	64	33%	NS
	5FU + ADR	61	32%	
(43)	Surgery alone	66	26%	p = 0.025 ; Bias ?? very low survival for control group
	MMC	68	41%	
(11)	Surgery alone	159	~ 41%	NS ; increased Progression free survival ; toxicity +
	5FU + ADR + MMC	155	~ 43%	
(12)	Surgery alone	100	NS	
	5FU + ADR + MMC	93		

(44)	Surgery alone	42	29%	NS ; Small study
	5FU + MMC + EpiADR	42	36%	
(45)	Chirurgie seule	55	13%	p < 0.01 ; Low follow-up (36 months), small study
	5FU + AF + EpiADR	48	25%	
			S. à 3 ans	
(17)	MMC	45	44%	p = 0.04 ; low power and small study
	MMC + UFT	40	67%	
(18)	Surgery alone	76	36%	p= 0.04 ; one mitomycineC and 3 months UFT
	MMC + UFT	72	56%	
(16)	Surgery alone	285	83%	NS ; Only tumor stage T1 and T2 in this study
	MMC + 5FU for 3 weeks followed by 18 months UFT	288	86%	
(15)	Surgery alone	137	48%	NS ; 2 toxic deaths
	5FU + AF suivi de EpiADR + VP16 + CDDP	137	52%	
(13)	Surgery alone	133	43%	NS ; One toxic death
	5FU une cure puis 5FU + CDDP 4 cures	127	48%	
(35)	Surgery alone	603	41%	p = 0.03; Non optimal surgery in 54%
	5FU + AF + radiothérapie		52%	
			S. 3 years	

**Abbreviations :** 5FU = 5-fluorouracile ; VLB = vinblastine ; CPM = cyclophosphamide ; MeCCNU = methyl-CCNU ; lev = levamisole ; ADR = adriamycine ; MMC = mitomycine C ; EpiADR = epiadriamycine ; UFT = tegafur ; VP16 = etoposide ; CDDP = cisplatine ; AF = acide folinique.

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## **Preoperative approaches including preoperative chemoradiation therapy in patients with potentially resectable gastric carcinoma.**

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The outcome of patients with local-regional gastric carcinoma is dismal. Only 40% to 50% of patients in the western world can have a “curative” (R0) resection because most patients are diagnosed in advanced stages. Recent Intergroup trial (0116) has demonstrated that postoperative chemoradiotherapy appears to prolong disease-free and overall survival of patients compared to the same parameters for patients who were observed following surgery (NEJM September, 06, 2001). Nevertheless, postoperative therapy can be afforded to only a select group of patients. Preoperative strategy can be exploited to treat more patients with locally advanced gastric carcinoma than postoperative strategy. Thus the concept of preoperative therapy to improve the rate of R0 resection has been attractive. Others and we have conducted a number of clinical trials in patients with potentially resectable gastric carcinoma. This strategy has clearly been feasible and the results have been encouraging. Following are the overall results:

<b>Authors (Et al.)</b>	<b>Regimen</b>	<b>Preop/ Postop Chemo</b>	<b>No. Patients</b>	<b>Clinical Response</b>	<b>Operated</b>	<b>R0 Resection</b>	<b>Pathologic CR</b>	<b>Median Survival (months)</b>
Ajani	EFP	2/3	25	24%	100%	72%	0%	15
Ajani	EAP	3/2	48	31%	85%	77%	0%	15.5
Leichman	CLF	2/2 (IP)	50	48%	94%	72%	6%	30+
Ajani	CFI	5/0	30	34%	97%	83%	6%	28
Lowy	FU/XRT (45 Gy)	0/IORT	24	NA	79%	79%	8%	NA

We have also utilized the strategy of preoperative chemoradiotherapy in this group of patients. One multi-institutional trial has been completed using an enhanced clinical staging (laparoscopy for peritoneal staging and endosonography (EUS) to define T and N stages). Patients with T2-3, anyN, and M0 stages were eligible. The strategy of this trial can be described as follows: **Step 1:** Patients first received two courses of chemotherapy (CT) with 5-FU (200 mg/m<sup>2</sup>/d as cont. inf. on d 1-21), folinic acid (20 mg/m<sup>2</sup> i.v. bolus on d 1,7,14, and 21) & cisplatin (20 mg/m<sup>2</sup>/d as i.v. bolus on d 1-5). The second course was repeated in 28 d.

**Step 2:** Following CT, patients began chemoradiotherapy (CTRT) with 45 Gy (25 Fxs in 5 weeks to the primary and regional nodes) and concurrent 5-FU (300 mg/m<sup>2</sup>/d as cont. inf. 5 d/wk during RT). **Step 3:** Five or six weeks after CTRT, patients were restaged and underwent an attempted resection. Following surgery, no further therapy was planned. Thirty-four patients have been enrolled. One patient withdrew consent, 3 developed M1 disease, and two died before surgery. Among 28 patients taken to surgery, 10 had a **pathCR** and 3 had a **pathPR** ( $\geq 90\%$  necrosis).

<b>EUS vs. Surgical Path</b>	
<b>• 21 Comparisons</b>	
<b>EUS Findings</b>	<b>Surgical Path</b>
<b>17 with T3</b>	<b>4 with T3</b>
<b>13 with N1</b>	<b>4 with N1</b>

Grade 4 toxicities occurred in 28% patients. There has been one postoperative death; otherwise the morbidity has been acceptable.

The currently ongoing trial uses Taxol-based induction chemotherapy and chemoradiotherapy. In this trial 31 patients have been registered. One patient died of sudden cardiopulmonary death prior to surgery, one developed M1 cancer before surgery, and 3 others had M1 cancer found at surgery. Among the 24 patients taken to surgery in the current trial, 7 had a **pathCR** and 3 had a **pathPR**. These preliminary results suggest that the unique strategy of preoperative chemoradiotherapy is feasible in patients with potentially resectable gastric carcinoma. A potential phase III trial is under discussion by the Intergroup.

## **Treatment of metastatic gastric cancer. Present and future**

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The chemotherapy of advanced gastric cancer has been for long time only marginal effective. The first generation protocol consisted of FAM which has been developed in the early seventies. The second generation protocol FAMTX which has been developed in the eighties has been proven to be superior in terms of improved response rate and survival. Other second generation protocols like Cisplatinum/ 5FU, EAP or ELF had comparable activity without major improvement. These studies motivated to strongly investigate further new protocols and in particular the use combination chemotherapy to increase survival in locally advanced inoperable tumors. In particular, the EAP-regimen was investigated in this subgroup of patients and has led to a substantial tumor reduction with the possibility for secondary resection. These studies have introduced the neoadjuvant chemotherapy followed by secondary surgery in locally advanced gastric cancer resulting in 10-20% long term survival.

Beyond this more recently third generation protocols have been developed and prospectively investigated. Presently the protocol of Epirubicin/ Cisplatinum/ continuous 5FU (ECF) or Mitomycin/ Cisplatinum/ continuous 5FU (MCF) are equally effective and proven superior to the FAMTX regimen. Furthermore, the combination of weekly infusion of 5FU/ high dose Folinic acid plus biweekly Cisplatinum (PFL) has been proven in the recent EORTC study to be associated at least with a 50% response rate and prolonged progression free and overall survival. ECF and PFL are probably the current standards for this patient population. The substitution of Cisplatinum by Oxaliplatin in the FOLFOX-regimen achieves the same efficacy, with reduced toxicity and easy outpatient management. This could be in particular of interest for patients with poor performance status. Current protocols investigate the substitution of infusional 5FU by Capecitabine or UFT/ Folinic acid or S1.

Further very active agents are Irinotecan, Docetaxel and Paclitaxel. Current protocols investigate double or triple combinations including these agents. Although, there is presently no indication that these fourth generation protocols are highly superior to the current standard. However, the large amount of active agents and the possibility of new multiple combinations, the introduction of new signal transduction inhibitors against Her2 and EGF-receptor or Cyclooxygenase-inhibitors is an excellent perspective for further improving the

outcome of this disease. Active participation in clinical trials to answer these many questions is mandatory.

## **Molecular pathology of exocrine pancreas cancer**

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Exocrine pancreas cancer is a devastating disease and there has been little clinical progress in its management: only 20% of patients with a 2 cm diameter tumor live more than 2 years. On the other hand, research carried out over the last decade has provided exciting insights into the molecular mechanisms underlying its development and progression.

More than 90% of exocrine pancreas tumors correspond to the "common ductal adenocarcinoma". Although the precise cell type from which these tumors originate is not known, there is extensive evidence suggesting that such cells are located in the pancreatic ducts. The most common genetic alterations associated with pancreatic cancer are mutations in *K-ras* (approximately 75%), mutations and allelic losses at the *p53* (17p13) and *p16* (9p21) loci, and homozygous deletions and mutations at the *Smad4* locus (18q21). The *p53*, *p16*, and *Smad4* genes appear to be inactivated in approximately 60%, 100%, and 50% of tumors, respectively. Mutations, genetic amplifications, and allelic losses have been reported at other loci but they appear to be less prevalent. A morphological reevaluation of proliferative lesions occurring in pancreatic ducts has recently led to their reclassification as PanIN (Pancreatic Intraductal Neoplasia) types 1, 2, and 3 as well as to a model of tumor progression. Genetic analysis of these lesions has shown that *K-ras* mutations occur early during the course of tumor development, such that their precise biological significance is at present obscure. By contrast, *p53* and *Smad4* inactivation appear to occur almost exclusively in PanIN-3 lesions (carcinoma in situ) showing greater potential for neoplastic progression. In addition to these genetic changes, epigenetic events are likely to be required for tumor progression. Among them are the overexpression of growth factors, growth factor receptors, and proteases, the loss of epithelial features of tumor cells, and the silencing of genes that negatively regulate tumor-promoting events. The precise contribution of two pathological hallmarks of the disease, a strong desmoplastic reaction and early perineural invasion, to its aggressive biological behaviour is not known but deserves thorough investigation.

Familial aggregation of pancreatic cancer occurs in 3-10% of cases and has been mainly associated to Familial Atypical Mole and Malignant Melanoma (*p16*), Familial Adenomatous Polyposis (*APC*), Hereditary non-polyposis Colon Cancer (*MLH1* and *MSH2*), Li-Fraumeni syndrome (*p53*), familial breast cancer (*BRCA2*), and hereditary chronic pancreatitis.

These exciting findings offer extraordinary opportunities for basic and clinical research, yet their current impact on the clinical management of patients at risk for pancreas cancer development, or with a clinical suspicion of harboring a tumor, is still limited.

## **Cáncer de páncreas exocrino. Epidemiología molecular y prevención**

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El cáncer es un conjunto complejo de procesos que resultan de la interacción de factores genéticos y ambientales. Se entiende por “ambiental” el medio o contexto físico, sanitario, cultural, económico y político. Creemos que los *estudios sobre las bases moleculares del cáncer*: i) es conveniente extenderlos más allá del laboratorio y continuarlos en el contexto clínico y poblacional, utilizando una metodología rigurosa (generalmente “epidemiológica”); ii) es conveniente acercarlos a la cabecera del enfermo y evaluar científicamente si pueden resultar útiles para la asistencia clínica; iii) a veces se pueden integrar con los estudios sobre las causas ambientales del cáncer, y valorar en qué medida pueden contribuir a mejorar la efectividad de los programas de prevención. También pensamos que el llamado “cisma” entre ciencias básicas, clínicas y de salud pública es perjudicial para los tres “mundos” o niveles, tanto en términos prácticos como de conocimiento científico [1].

*Conocer las causas* del cáncer de páncreas exocrino es quizás el objetivo científico más ambicioso en la investigación de dicha neoplasia, tanto desde un punto de vista biológico como desde la perspectiva de la salud pública. Por dos razones principales:

1. Por una parte, porque dicho conocimiento es *un pre-requisito para la prevención primaria*. Ésta es la única forma de prevención que permite disminuir la incidencia de la enfermedad (el número de casos nuevos en una población y tiempo definidos), en contraste con la prevención secundaria (la cual, por definición, sólo puede aplicarse a casos ya existentes, y que en la práctica resulta infrecuente en el cáncer de páncreas).
2. Por otra parte, porque las *interacciones genético-ambientales* constituyen un campo etiopatogénico especialmente relevante en el caso de ésta y de otras neoplasias. El estudio de tales interacciones es una oportunidad excelente para alcanzar nuevos conocimientos científicos sobre las bases moleculares del proceso carcinogénico.

Así, pues, las investigaciones integrativas sobre las causas del cáncer son un posible *punto de encuentro natural* entre la biología molecular, la medicina preventiva y la epidemiología.

1. Porta M, et al La búsqueda de factores de riesgo para el cáncer de páncreas: práctica, paciencia y paradigmas.  
*Gastroenterología & Hepatología* 1997; 20: 259-273.

## **Exocrine pancreatic cancer. Adjuvant treatment.**

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From the standpoint of clinical trials on the adjuvant treatment of adenocarcinoma of the exocrine pancreas, patients with potentially resectable disease are particularly "bad actors" for a number of reasons: the number of cases with non radical resection at surgery is substantial in several centers; the accuracy in determining tumor free margin at the retroperitoneal resection site is poor; the complication rate of pancreatic surgical interventions is very high; recovery is often delayed well beyond the usual time to start adjuvant treatment programs; the distinction between true pancreatic cancer and cancer of the perampullary region (bearing a better prognosis) may be difficult. Even when patients are selected for enrollment in postoperative adjuvant programs, 20% of them may be expected not to start the planned therapy. This is the explanation as to why the knowledge of the efficacy of adjuvant treatment in this disease is so limited. The highest number recruited in a randomized adjuvant study is 285 in the ESPAC trial recently reported at the 2000 ASCO meeting. This trial showed no benefit in terms of survival from either chemotherapy alone or RT+ chemotherapy or RT+ chemotherapy followed by chemotherapy over surgery alone. In this context it is surprising that so many centers regard postoperative chemotherapy +RT as standard adjuvant treatment, based on the GITSG experience only (median survival of chemotherapy +RT treated patients was almost double that of untreated control, 18 vs 11 months, on a total of 52 patients only).

There are two other factors rendering prohibitive this field of clinical investigation. The first is the notion of the very short time from local to distant failure when the disease recurs, that explains why aggressive attempts at optimizing local control with either intraoperative RT or preoperative chemotherapy plus RT do not substantially impact on the overall survival of these patients. The second is the lack of active new agents. In this respect, the slightly higher activity of gemcitabine compared to fluorouracil in the advanced setting justifies the conduct of the currently ongoing RTOG adjuvant study comparing gemcitabine or FU administered either before or after FU based chemotherapy plus RT.

## **Locally advanced and metastatic disease: present approach and future perspectives**

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In pancreatic cancer, early stages account for fewer than 20% and overall survival in advanced disease is less than 1% at 5 years. Therefore the development of new agents and/or combinations in search of an active chemotherapy is urgently needed, also because intensive regimens seem to be contraindicated in this disease (GISCAD 1998).

Gemcitabine (GEM) is the first agent able to improve Quality of Life (QoL) and Performance Status (PS) (Casper 1994, Rothenberg 1996 and Burris 1997) and to increase the very limited results obtained with the standard drug 5-fluorouracil (5FU). A clinical benefit (CB) was observed in 25-30% patients and this positive effect was reproducible also outside clinical trials (Storniolo 1999). Also a prolonged survival versus 5FU was achieved (1 year: 18% vs 2%) (Burris 1997). The role of GEM administered at a fixed rate (10 mg/min), as proposed by Tempero in 1999, is not yet completely elucidated (GISCAD 2000).

In phase II trials both the combination of GEM + 5FU (Hidalgo 1999 with CI 5FU, GISCAD 1999 with bolus 5FU) and the association of the same drug + cisplatin (Colucci, 2001) were promising, but a well-defined superiority in comparison to GEM alone has not yet been established (Di Costanzo 2001). A four-drug regimen (PEF-G) was reported as very active, but in a fairly selected population (Reni 2001).

The combination of GEM and other drugs is now under scrutiny in some phase III trials:

- 1) GEM + 5FU bolus versus GEM: preliminary data disappointing (Berlin, ECOG 2001)
- 2) GEM + CDDP versus GEM: ongoing trial with acceptable toxicity (Heinemann 2001)
- 3) GEM + CDDP versus GEM: recently started "pragmatic" trial (Italian Intergroup study)
- 4) GEM + OHP (GEMOX) versus GEM: ongoing French-Italian (GERCOD/GISCAD) study. This study is based on the promising results reported by GERCOD at ASCO (Louvret and De Gramont 2001)

Another field of interest in pancreatic cancer is the evaluation of new classes of drugs. Matrix metalloproteinases (marimastat and others) were so far disappointing, as well as farnesyl-transferase inhibitors, but trials are still ongoing. Also monoclonal antibodies (herceptin, IMC-C225...) are being evaluated and we have some very preliminary but encouraging data (Safran 2001 and Abbruzzese 2001).

As a conclusion, we can say that the future for the treatment of this very aggressive disease seems to be brighter than reported before and that medical oncologists from every country are urged to enroll their patients in well-designed clinical trials, hopefully in the frame of a multinational collaboration.