

DNA Repair of Mismatched Bases and Early Molecular Changes in Tumorigenesis

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The overall goal of our research is two-fold: to uncover the molecular genetic alterations involved in the development of hereditary and sporadic human cancer, and to validate critical regulatory molecules/pathways as targets for innovative approaches of cancer prevention and therapy. Our main objectives are to characterize the genetic changes in cancer cells, and to evaluate normal gene function and pathogenesis of disease. Ultimately, knowledge about the molecular basis of tumor development will facilitate the establishment of more effective ways to prevent, diagnose and treat cancer.

All common epithelial cancers (or carcinomas) are characterized by the accumulation of a discrete number of genetic and epigenetic mutational hits. These rate-limiting mutations accompany the transition from a normal epithelium into benign tumor and later malignant tumor (multistep carcinogenesis). Invariably, some of these changes correspond to biallelic inactivation of the critical tumor suppressor gene that acts as “gate-keeper” of proliferation control in a given target epithelium. At the same time, all cancers are characterized by an increase in the mutation rate (or mutator phenotype), which suggests the involvement of “caretaker” genes governing the stability of the genome.

Colorectal cancer (CRC) is the paradigm of multistep carcinogenesis and constitutes the second leading cause of death from cancer in Western countries. The two main familial CRC syndromes – hereditary non-polyposis CRC (HNPCC, or Lynch Syndrome) and familial adenomatous polyposis (FAP) – represent models for the involvement of caretaker and gatekeeper genes, respectively.

HNPCC patients carry a germline mutation in one of the genes involved in long-patch DNA mismatch repair (MMR), a specialized system that corrects base-base mismatches, short insertions/deletions and recombination-derived heteroduplexes. MMR genes include *MSH* [mutS homolog] 2, *MLH* [mutL homolog] 1, *MSH3*, *MSH6*, *PMS* [postmeiotic segregation] 2 and *PMS1*. MMR inactivation leads to a 100- to 1000-fold increase in the mutation rate, resulting in predisposition to both CRC and extracolonic malignancies, such as cancers of the endometrium, stomach, ovary, brain, skin and urinary tract.

In an effort to identify novel genes involved in DNA repair or related DNA transactions, we employed the yeast interaction trap using MMR proteins as “baits”. One of the *MLH1* interactors that we identified is a novel human DNA repair protein, which we named *MED1*. *MED1* is involved in the repair of mismatched bases in a pathway of base excision repair. In fact, we and others have previously shown that *MED1* (also named *MBD4*) removes thymine and uracil from G:T and G:U



mismatches with its thymine and uracil DNA *N*-glycosylase activity. MED1 activity prefers G:T and G:U mismatches located in the context of methylated and unmethylated CpG sites. Since these mismatches originate via spontaneous hydrolytic deamination of 5-methylcytosine and cytosine to thymine and uracil, respectively, MED1 is likely involved in the protection of CpG sites from deamination events. In the absence of MED1-mediated repair, in the next round of DNA replication, the mismatched thymine and uracil will lead to incorporation of adenine. The end result will be G:C→A:T transition mutations, which in the context of changes at CpG sites, are the most frequent mutations in human cancer. We are currently investigating the biology and biochemistry of MED1 and its involvement in CpG site genomic stability and human cancer.

Whereas MMR proteins and MED1 act as caretakers, mutations in a “gatekeeper” of normal proliferation in the colon characterize familial adenomatous polyposis (FAP). FAP patients carry a germline mutation in one allele of the tumor suppressor gene *APC*, which regulates the size of the stem cell compartment in the colon and the orderly transition of proliferation into differentiation along the axis of colonic crypts. Somatic loss of the second *APC* allele in colonic cells leads to formation of hundreds or thousands of colorectal polyps (or adenomas), some of which may evolve into malignant cancer. In addition, FAP patients are predisposed to the development of gastric and duodenal polyps, desmoid tumors, osteomas, and hepatoblastomas.

We are currently analyzing morphologically normal colonic cells bearing a single-hit in the *APC* tumor suppressor gene (heterozygous state) in an effort to identify the earliest molecular changes in colorectal tumorigenesis, prior to adenine formation. This novel approach is based on the hypothesis that while biallelic inactivation of the gatekeeper tumor suppressor gene is necessary to initiate tumorigenesis of a given target epithelium, single-hit alterations of this gene might be associated with initial molecular alterations (pre-initiation) present in the “normal” mucosa. These early changes may represent molecular targets for strategies of intervention based on novel chemopreventive agents.

The role of MED1 in the cellular response to DNA damage. Cortellino, Giri, Bassi, Lurie, Bellacosa, in collaboration with Kinsella,^a Schupp,^a Larue,^b Meropol[§]

We recently uncovered a novel role of MED1 in the DNA damage response to anti-tumor agents. DNA damaging agents cause cell killing by activating checkpoints that lead to cell cycle arrest and, in the presence of excess, unrepairable damage, to apoptosis. We recently showed that *Med1*-null mouse embryonic fibroblasts (prepared from mice with targeted inactivation of the *Med1* gene generated in our laboratory), much like MMR-deficient cells, are resistant to alkylating agents and other DNA damaging anti-tumor drugs. This phenotype is due to a defect in checkpoint activation and is associated with reduced steady state amounts of several of the early MMR proteins, in particular MLH1 and MSH2 (Cortellino et al., *PNAS* 100: 15071, 2003).

We are currently investigating whether MED1 also modulates sensitivity to other types of DNA damage, namely that induced by ionizing radiation. For these experiments, *Med1*-null and control cells are treated with gamma irradiation, alone or in combination with radiosensi-

tizer drugs. In parallel experiments, we are analyzing the role of MED1 in the signaling pathways initiated by DNA damage.

Kinetic characterization of MED1 glycosylase activity: preference for halogenated pyrimidines and involvement in the cytotoxicity of 5-iododeoxyuridine. Cortellino, Bellacosa, in collaboration with Loh,^a Schupp,^a Kinsella,^a Turner^c

As mentioned in the introduction, MED1 acts as a thymine and uracil DNA *N*-glycosylase on T:G and U:G mismatches that occur at CpG methylation sites due to spontaneous deamination of 5-methylcytosine and cytosine, respectively. In order to elucidate the mechanisms that underlie sequence discrimination by MED1, we performed single turnover kinetics with the isolated, recombinant glycosylase domain of MED1. This allowed quantification of MED1 substrate hierarchy, confirmed MED1 preference for mismatches within CpG context, and demonstrated its preference for hemimethylated base mismatches. Furthermore, the k_{st} values obtained with the uracil analogues 5-fluorouracil and 5-iodouracil were over 20–30-fold higher than those obtained with uracil, indicating substantially

higher affinity for halogenated bases. A 5-iodouracil precursor is the halogenated nucleotide 5-iododeoxyuridine (5IdU), a cytotoxic and radiosensitizing agent. Cultures of mouse embryo fibroblasts (MEFs) with different *Med1* genotype derived from mice with targeted inactivation of the *Med1* gene (Cortellino et al., *PNAS* 100:15071, 2003) were evaluated for sensitivity to 5IdU. The results revealed that

Med1-null MEFs are more sensitive to 5IdU than wild-type MEFs in both MTT and colony formation assays (Figure 1). Furthermore, HPLC analyses revealed that Med1-null cells exhibit increased 5IdU incorporation in their DNA. These findings establish MED1 as a *bona fide* repair activity for the removal of halogenated bases and indicate that MED1 may play a significant role in 5IdU cytotoxicity.

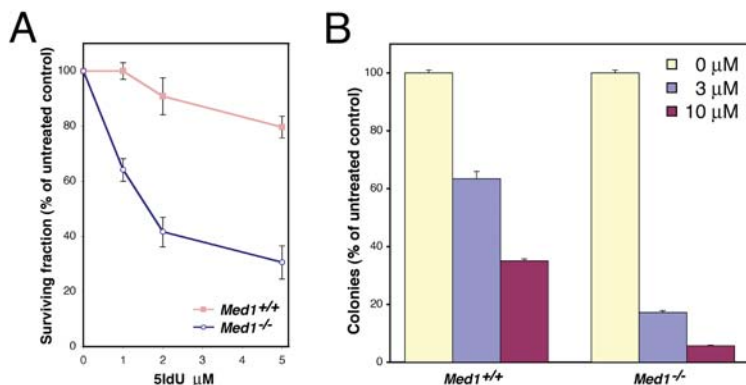


Figure 1. Med1 status affects sensitivity to 5-iododeoxyuridine (5IdU). **A)** Survival analysis conducted with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay. Wild-type and Med1-null MEFs were treated with the indicated doses of 5IdU for 72 hours and the surviving fraction was detected by measuring the absorbance of metabolized MTT. **B)** The sensitivity of cells to 5IdU was tested by colony assay formation. Wild-type and Med1-null MEFs were treated with the indicated doses of 5IdU for 48 hours and then allowed to form colonies in drug-free medium for 7 days. Colonies were stained with Crystal blue and their number was plotted with respect to untreated control cultures.

Thymine glycol glycosylase activity of MED1.

Bassi, Bellacosa, in collaboration with Turner,^C Canutescu,[§] Chan,[§] Dunbrack,[§] Myers,[§] Seeholzer,[§] Yeung[§]

Oxidative damage of 5-methylcytosine at CpG sites may generate thymine glycol (Tg). Based on the proposed role of MED1 in CpG site protection, we investigated the possibility that MED1 may display thymine glycol glycosylase activity for G:Tg pairs at CpG sites. Recombinant human MED1 protein was incubated with ³²P-labelled double-strand oligonucleotides containing the *cis*-5R,6S stereoisomer of Tg in various sequence contexts. The Tg-containing oligonucleotide was synthesized by using a commercially available *cis*-5R,6S Tg phosphoramidite and its identity confirmed by mass spectrometry. Results showed that MED1 displays thymine glycol glycosylase activity for G:Tg pairs at CpG sites. No activity was detected on A:Tg (i.e., the product of thymine oxidation). Analysis of the sequence context

revealed that MED1 prefers G:Tg pairs in CpG sites. These results suggest that MED1 may protect the stability of CpG sites not only from deamination events but also from oxidative damage.

Hypermethylation of the *MED1* promoter and analysis of the *MED1* gene in cancer. Cortellino, Bellacosa, in collaboration with Aaltonen,^d Arnoletti,^e Fodde,^f Frolov,^e Genuardi,^g Neri,^h Percesepe,ⁱ Ponz de Leon,^j Riccio,^k Viel,^l Godwin[§]

By counteracting mutagenesis caused by spontaneous deamination of 5-methylcytosine and cytosine, the *MED1* DNA repair gene may act as a tumor suppressor gene of the “caretaker” type, providing genomic fidelity at CpG sites. In keeping with this possibility, we previously detected *MED1*-inactivating mutations and loss of heterozygosity at the *MED1* locus in colorectal, endometrial and pancreatic tumors characterized by microsatellite instability (Riccio et al., *Nature Genet.* 23:266, 1999). By analogy

with other DNA repair genes, such as MLH1, we hypothesized that hypermethylation of the *MED1* promoter may lead to decreased MED1 protein expression and thus impaired G:T and G:U mismatch repair during tumorigenesis. Methylation status of the *MED1* promoter was investigated in a panel of ovarian and colorectal cancer cell lines by sequence analysis following bisulfite treatment. Methylation frequency of the involved CpG sites was quantified by methylation-specific PCR of subclones. The clinical relevance of *MED1* promoter methylation was further investigated in colorectal cancer specimens by methylation specific PCR comparing matched normal mucosa, adenomas, and carcinoma. MED1 protein expression was evaluated by western blot. The results revealed that some CpG sites in the *MED1* promoter are frequently and preferentially methylated ($\geq 50\%$) in ovarian cancer cell lines with low/reduced MED1 expression. Treatment of colorectal cancer cell lines with demethylating agents reversed *MED1* methylation and restored protein expression. The same CpG sites were found to be more frequently methylated in colorectal cancer specimens when compared to matched normal colon mucosa; and expression of MED1 protein was consistently decreased in colorectal polyps and cancers when compared to normal colon mucosa. These findings indicate that the *MED1* gene is inactivated by promoter hypermethylation in ovarian and colorectal cancer.

In parallel experiments, we are conducting a complete mutational analysis of the *MED1* gene in several epithelial cancer types.

Analysis of *MED1* mutations in colorectal cancer patients from Eastern Cooperative Oncology Group (ECOG) clinical trials. Bassi, Bellacosa, in collaboration with Catalano,^m Cooper,[§] Meropol,[§] Yeung[§]

Due to the role of MED1 in both DNA repair and response to DNA damage induced by alkylating agents and other chemotherapeutic drugs used in the clinic, tumors with MED1 deficiency may also be resistant to agents commonly used in the treatment of colorectal cancer, such as 5-fluorouracil, irinotecan, and oxaliplatin. In order to test this hypothesis, we are conducting a mutational analysis of the *MED1* gene in colorectal cancer specimens obtained from patients enrolled in ECOG-sponsored clinical trials. The objectives of this

project involve to precisely evaluate the frequency of *MED1* mutations in colorectal cancer and to determine whether these mutations have an impact on response to therapy and prognosis.

Altered gene expression patterns in phenotypically normal cells from individuals heterozygous for mutations in tumor suppressor genes. Caretti, Bellacosa, in collaboration with Knudson,[§] Clapper,[§] Boman,ⁿ Campbell,[§] Cooper,[§] Coudry,[§] Crowell,^o Devarajan,[§] Godwin,[§] Henske,[§] Howard,[§] Kopelovich,^o Linehan,^o Nicolas,[§] Ross,[§] Stoyanova,[§] Testa,[§] Yeung[§]

By combining Knudson's "two-hit" and the multistep tumorigenesis theories, we hypothesized that while biallelic inactivation of the gatekeeper tumor suppressor gene is necessary to initiate tumorigenesis of a given target epithelium, single-hit mutations of this gene might be associated with initial molecular alterations (pre-initiation) present in the morphologically "normal" mucosa. In principle, these early changes would have the highest probability of showing a direct bearing on subsequent tumor induction, and the lowest probability of being marginal by-products of the neoplastic phenotype. Furthermore, they might represent molecular targets for strategies of intervention based on novel chemopreventive agents. In order to detect these changes, we conducted microarray studies of primary epithelial cultures from patients predisposed to cancer, which by definition carry a mutation in one allele of a tumor suppressor gene, and control individuals with intact copies of the tumor suppressor gene.

We studied three different sites: kidney (predisposing genotypes: *VHL* and *TSC1/2*) and breast/ovary (predisposing genotypes: *BRCA1/2*). A total of 270 RNA samples were processed, from primary renal, breast and ovarian epithelial strains (n=90 for each target organ). The breast and ovarian epithelial RNAs were obtained from cells cultured from 18 patients (*BRCA1* or *BRCA2* mutation carriers and wild-type controls; n=6 per group, acting as biological replicates). The renal epithelial RNAs were obtained from 18 patients (*VHL* or *TSC1/2* mutation carriers and wild-type controls; n=6 per group, acting as biological replicates). Using the Affymetrix platform, we obtained expression data on 54,675 probe sets for each sample. Affymetrix data were pre-processed

and normalized using the Robust Multi-chip Average (RMA) method and class comparison analyses were performed in order to identify differentially expressed genes among the different genotypes for each target organ. Several changes in gene expression were identified in the comparisons among genotypes, suggesting that heterozygous mutations in *BRCA1* and *BRCA2* (for breast and ovary) and in *VHL* and *TSC1/2* (for kidney) affect expression profiles of primary epithelial cells of the respective target organs.

These findings demonstrate that heterozygosity for a mutant tumor suppressor gene may alter the expression profiles of phenotypically normal epithelial cells in a gene-specific manner, confirming our earlier findings in renal cells (Stoyanova et al., *Cancer Biol. Ther.* 3: 1313, 2004). Detectable effects of “one-hit” represent early molecular changes in tumorigenesis that may serve as targets for chemopreventive intervention.

Microarray analysis of microdissected, morphologically normal colorectal mucosa from FAP and HNPCC patients. Caretti, Bellacosa, in collaboration with Clapper,[§] Knudson,[§] Boman,[†] Cooper,[§] Coudry,[§] Crowell,[○] Devarajan,[§] Kopelovich,[○] Ross,[§] Seeholzer,[§] Stoyanova,[§] Yeung[§]

The same genetic strategy outlined in the previous project is being applied to individuals predisposed to colon tumors (predisposing genotypes: *APC* and *MLH1*). It is difficult to establish in culture primary epithelial cells from the colon. For this reason, in order to uncover the gene expression changes induced by the single-hit *APC* or *MLH1* mutation, we are conducting a microarray analysis on biopsies of morphologically normal (non-tumorous) colonic mucosa sampled during colonoscopy. Samples are microdissected by laser capture microdissection (LCM) in order to separate individual colonic crypts consisting of pure epithelial cells, free from the surrounding stromal cells.

Using the Affymetrix platform, we are comparing the gene expression profile of morphologically normal colonic epithelial cells from: 1) FAP patients (*APC* mutation carriers); 2) FAP patients undergoing treatment with the chemopreventive agent sulindac; 3) HNPCC patients (*MLH1* mutation carriers); and 4) control individuals with intact *APC* and MMR system, and

negative personal/familial history of cancer. We hypothesize that treatment with chemopreventive agents may reverse or counteract some of the changes induced by a mutant tumor suppressor gene causing a reprogramming of gene and protein expression/distribution that can be comprehensively monitored with microarray, and possibly proteomics, techniques.

Expression profiling of hyperplastic polyps of the colon. Caretti, Bellacosa, in collaboration with Cooper,[§] Coudry,[§] Stoyanova[§]

Another approach towards the identification of early genetic changes during colorectal tumorigenesis involves the molecular characterization of hyperplastic polyps and aberrant crypt foci of the colon. Hyperplastic polyps and aberrant crypt foci have been identified as early lesions characterized by morphological features of increased cell number in colonic crypts and the potential to progress to adenoma and carcinoma. In this project, we are conducting a comprehensive molecular-morphological approach in order to characterize in detail these lesions on the basis of their mRNA expression profile.

The overall hypothesis of the proposed studies is that alterations in gene transcription drive a morphogenetic process that leads to a change of the normal colorectal mucosa into hyperplastic polyps. Since pathological and epidemiological observations suggest that hyperplastic polyps and aberrant crypt foci can progress to adenomas and carcinomas, it is likely that alterations in the expression profile of these precursor lesions, in comparison to normal mucosa, are relevant and predispose to subsequent malignant progression. Characterization of these early molecular changes by microarray technologies will allow the identification of novel molecular targets for future pharmacological and environmental intervention.

Role of the protein kinase AKT in epithelial mesenchymal transition, motility and invasiveness. Xu, Caretti, Bellacosa, in collaboration with Grille,^b Larue,^b Cooper,[§] Clapper,[§] Chang,[§] Hensley,[§] El-Deiry^p

The oncogenic serine/threonine kinase AKT (also known as PKB) is a downstream effector of the phosphatidylinositol 3' kinase (PI3K) and is frequently activated in human cancer (1). Recently, using squamous cell carcinoma lines engineered to express constitutively-active

AKT, we showed that AKT activation causes epithelial-mesenchymal transition (EMT) [Grille et al., *Cancer Res.* 63:2172, 2003; (2)]. EMT is an important process during development and oncogenesis by which epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility. Indeed, cells expressing constitutively-active AKT exhibited reduced cell-cell adhesion, increased motility on fibronectin-coated surfaces, and increased invasiveness in an animal model of invasion.

We are currently investigating the mechanisms and the signal transduction pathways by which AKT causes EMT. We are also investigat-

ing the transcription profile of cells undergoing EMT upon AKT activation. In parallel experiments, we generated transgenic mice expressing constitutively active alleles of AKT and PI3K in the colonic mucosa, in order to examine the role of AKT activation in colorectal tumorigenesis. Both optical and MRI imaging are used for the detection and monitoring of tumors during progression.

Since AKT is activated in many human carcinomas, and the AKT-driven EMT may confer the motility required for tissue invasion and metastasis, these studies may lead to future therapies based on AKT inhibition to control tumor cell invasion and metastasis.

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