

Early Detection and Staging of Cancer by Translational Molecular Genetic Analysis

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The primary focus of our research program is the application of basic knowledge of the molecular genetics and epigenetics of urological (prostate, kidney and bladder), breast and ovarian cancer to translational research, particularly in regard to the molecular detection of cancer. Since surgical resection is the current mainstay of cancer treatment, novel and well-conceived approaches to the early detection of cancer are urgently needed. The clonal genetic and epigenetic alterations that initiate and drive tumorigenesis are very promising markers for molecular detection because they can precede obvious cancer, can be detected by PCR-based techniques at very sensitive levels, and are highly specific as they represent an absolute, rather than quantitative, change. We have designed and tested novel, highly sensitive molecular assays to detect cancer specific alterations in tumor cells, cell debris or free DNA released from apoptotic and necrotic cells into bodily fluids that surround or drain from the organ of interest e.g. urine and serum. To date, we have mainly focused on early detection tests although, we increasingly see possibilities for simultaneous molecular staging and prognosis, and even the ability to design a profile for personalized treatment of an individual patient's cancer. We are particularly interested in further identifying and understanding epigenetic alterations important in the biology of the cancer cell, and with translational application for risk assessment, early diagnosis, prognostic monitoring, treatment, and the prevention of cancer.



Molecular detection of bladder cancer in urine.

Dulaimi,* Ibanez, Cairns, in collaboration with T. Al-Saleem,[§] Uzzo,[§] Greenberg,[§] Chen[§]

Silencing of tumor suppressor genes (TSG), such as *p16^{INK4a}* the mismatch repair gene *hMLH1* and *BRCA1* have established hypermethylation as a common mechanism for TSG inactivation in human cancer. Because hypermethylation is frequent, occurs early in tumorigenesis, can be detected at sensitive levels by PCR, and can provide a "yes or no" or quantitative answer for the presence of cancer cell DNA and is therefore potentially highly specific, it is a very promising target for molecular detection. We have pioneered hypermethylation-based detection of prostate and kidney cancer in urine and have now extended our work to bladder cancer. Bladder cancer is potentially curable in the majority of cases; however, the prognosis for patients with advanced disease at presentation remains poor. Current non-invasive

tests such as urine cytology lack sufficient sensitivity to detect low grade, low stage tumors. Methylation specific PCR (MSP) can determine the presence or absence of methylation of a gene locus at a sensitivity level of 1 methylated allele in 1000 unmethylated alleles, appropriate for identifying cancer cell DNA in a body fluid such as urine.

We tested for detection of the *APC*, *RASSF1A* and *p14^{ARF}* TSG in tumor and matched sediment DNA from urine specimens obtained prior to surgery from 45 bladder cancer patients, as well as normal and benign control DNAs. Hypermethylation of at least one of three genes was found in all 45 tumor DNAs (100% diagnostic coverage). We detected gene hypermethylation in the matched urine DNA from 39 of 45 patients (87% sensitivity) including 16 cases that had negative cytology. No hypermethylation of *APC*, *RASSF1A* or *p14^{ARF}* was observed in normal transitional cell DNAs

or in urine DNAs from normal healthy individuals and patients with inflammatory urinary disease. Furthermore, an unmethylated gene in the tumor DNA was always found to be unmethylated in the matched urine DNA (100% specificity). Hypermethylation of TSG is common in bladder cancer and was found in all grades and stages of tumors examined. MSP may enhance early detection of bladder cancer using a non-invasive urine test. Ultimately, molecular detection using a methylation panel in urine may yield simultaneous diagnostic and prognostic information for prostate, kidney and bladder cancer.

Identification of novel cancer genes by a global epigenetic reactivation screen of kidney cancer. Ibanez, Dulaimi,* Hoffman, Cairns

Aberrant promoter hypermethylation is a common mechanism for inactivation of tumor suppressor genes (TSG) in cancer cells. To generate a global profile of genes silenced by hypermethylation in renal cell cancer (RCC), we performed an expression microarray-based analysis of genes reactivated in the 786-0, ACHN, HRC51 and HRC59 RCC lines after treatment with the demethylating drug 5Aza-2 deoxycytidine (5Aza-dC) and histone deacetylation inhibiting drug trichostatin A (TSA). Between 111 and 170 genes were found to have at least 3-fold upregulation of expression after treatment in each cell line. To establish the specificity of the screen for identification of genes epigenetically silenced in cancer cells, we validated a subset of 12 upregulated genes. The promoter methylation status and transcription status of the 12 genes were validated by semi-quantitative RT-PCR of untreated and treated cell line cDNA and by bisulfite sequencing and methylation specific PCR (MSP) of tumor and normal cell DNA. Three of the 12 genes (*IGFBP1*, *IGFBP3* and *COL1A1*) showed promoter methylation in tumor DNA but were unmethylated in normal cell DNA; 1 gene (*GDF15*) was methylated in normal cells but more densely methylated in tumor cells; and 1 gene (*PLAU*) showed cancer cell specific methylation that did not correlate well with expression status. The remaining seven genes had unmethylated promoters; however, there is evidence for at least one of these genes (*TGM2*) to be regulated by another gene, *RASSF1A*, that

was indeed methylated in the RCC lines. Thus, we were able to demonstrate that upregulation of at least 6 of the 12 genes examined was due to epigenetic reactivation. The *IGFBP1*, *IGFBP3* and *COL1A1* gene promoter regions were found to be frequently methylated in primary renal cell tumors and further study will likely provide insight into the biology of the disease and facilitate translational studies in renal cancer.

Methylation-based detection of ovarian cancer in serum. Ibanez, Dulaimi,* Cairns, in collaboration with Edelson,§ Bergman,§ Hyha§

Because existing surgical and management methods can consistently cure only early stage ovarian cancer, novel strategies for early detection are required. The genetic and epigenetic alterations that initiate and drive tumorigenesis can be used as targets for the detection of cancer in clinical specimens such as bodily fluids. Since some genetic and epigenetic events will occur early in the disease process, molecular diagnosis may allow detection prior to symptomatic or overt radiographic manifestations. Several cancer genes of clear biological significance, including *p16^{INK4a}* and *BRCA1*, have been found to have hypermethylation of normally unmethylated CpG islands within the promoter region in ovarian cancer cells.

Using MSP, we screened matched tumor and pre-operative serum/plasma DNAs obtained from 50 patients with ovarian tumors for hypermethylation status of the normally unmethylated *BRCA1* and *RAS association domain family protein 1A (RASSF1A)*, *adenomatous polyposis coli (APC)*, *p14^{ARF}*, *p16^{INK4a}* or *death associated protein-kinase (DAP-Kinase)* tumor suppressor genes. Hypermethylation of one or more of the gene panel was found in all 50 tumor DNAs (100% diagnostic coverage). Hypermethylation was observed in all histological cell types, grades and stages of ovarian tumor examined. An identical pattern of gene hypermethylation was found in the matched serum DNA from 41 of 50 patients (82% sensitivity) including 13 of 17 cases of Stage I disease. In contrast, no hypermethylation was observed in non-neoplastic tissue or serum from control women. We conclude that promoter hypermethylation is a common and relatively early event in ovarian tumorigenesis that can be detected in the serum DNA from patients with ovary-confined (Stage IA or B) tumors.

Breast cancer detection in serum using gene hypermethylation. Dulaimi,* Ibanez, Cairns, in collaboration with Hillinck,^b T. Al-Saleem[§]

Although breast cancer mortality in the U.S. is declining and the proportion of cancers detected early is increasing, there is more benefit to be realized from screening. Thus, novel, well-conceived strategies for early detection are extremely important. Hypermethylated regions of tumor suppressor genes represent excellent targets for novel diagnostic approaches based on PCR. Several tumor suppressor genes have been found to be methylated at a sufficiently early point in breast tumorigenesis when treatment can result in a better outcome (e.g., DCIS and Stage I tumors). Serum/plasma is a readily accessible bodily fluid and provision of a specimen does not require the presence of a specialist. DNA is known to be released into serum/plasma which is enriched for tumor DNA in cancer patients. We have delivered the first proof of principle of sensitive and specific early detection of breast cancer in serum/plasma DNA using a hypermethylated gene panel.

Our aim is now to validate these findings in a larger set of tumors and paired pre-operative serums from early stage disease patients, as well as normal control serums, with quantitative real time methylation specific PCR technology. This work will determine the optimal hypermethylated gene panel, centered upon the *APC*, *RASSF1A* and *DAP-Kinase* tumor suppressors, in respect to diagnostic coverage, sensitivity and specificity. To determine if detection of methylation in serum can precede diagnosis of disease, validation is ongoing in a case control study of serum obtained from women at average or increased risk of breast cancer alongside women whom remain free of disease. Through

the development and application of this molecular technology, it may be possible to substantially improve the way we currently diagnose patients with this life threatening cancer.

Monitoring of tumor suppressor gene methylation in follow-up renal cancer patients.

Dulaimi,* Ibanez, Cairns, in collaboration with Uzzo,[§] Greenberg,[§] T. Al-Saleem[§]

We hypothesized that in patients with no clinical evidence of disease several weeks or months after undergoing nephrectomy for organ-confined disease and whose tumor and pre-operative urine showed hypermethylation, a follow-up urine specimen would show absence of hypermethylation to mirror the clinical absence of disease. We obtained a voided urine specimen, several weeks or months after nephrectomy, from 25 patients, the majority of whom had no clinical evidence of disease at time of follow up. Patients were aged 33 to 73 years; 15 patients had Stage I, 4 Stage II, and 4 Stage III cancers. Using MSP, we then examined the methylation status of a panel of genes in the tumor, pre-operative urine and post-operative urine DNA. Twenty-three of the follow-up urine DNAs showed only unmethylated alleles. The remaining 2 follow-up urine DNA samples showed methylation of the same gene methylated in the tumor. One of these patients had a partial nephrectomy with complete resection of a clear cell tumor as judged by pathologically negative surgical margins. The second patient had undergone a left partial and a right nephrectomy and may have hereditary cancer. Our study further supports the specificity of tumor suppressor gene promoter hypermethylation for the presence of cancer and, importantly, may prove to be a useful procedure for molecular monitoring of renal cancer.

Publications

Ibanez de Caceres, I., Dulaimi, E., Hoffman, A.M., Al-Saleen, T., Uzzo, R.G., Cairns, P. Identification of novel target genes by an epigenetic reactivation screen of renal cancer. *Cancer Res.* (in press).

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