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Alcohol Consumption and Breast Cancer

By Saijun Fan, MD, PhD

BREAST CANCER IS THE MOST COMMON CANCER AMONG WOMEN. RISK FACTORS ASSOCIATED with breast cancer include inherited mutation in the breast cancer susceptibility genes *BRCA1* and *BRCA2*; personal or family history of breast cancer; early onset of menstruation; late menopause; and never having had children or having a first child after age 30.

In recent years, epidemiological studies have suggested that alcohol consumption is associated with a high risk for breast tumorigenesis.[1-3] In a pooled analysis of six prospective cohort studies examining dietary factors in breast cancer, increased alcohol intake correlated significantly with breast cancer risk.[1] Moreover, the combination of alcohol consumption and postmenopausal estrogen replacement therapy synergistically enhanced the risk of cancer. In addition, several studies have reported that increased levels of circulating estrogen are associated with alcohol use; however, other studies have failed to demonstrate an increase in circulating or urinary estrogen in response to alcohol consumption.[4] Increase of mammary gland carcinogenesis, change of carcinogen metabolism, and impaired immune defense also contribute to alcohol's effects on breast cancer development and progression.[5]

Mechanism of Action

Alcohol is an etiologic agent for several different tumor types, including upper aerodigestive cancers (mouth, oropharynx, hypopharynx, and esophagus) and breast cancer. Alcohol is metabolized by the microsomal ethanol-oxidizing system, the activity of which is enhanced by chronic alcohol use. Alcohol is converted by alcohol dehydrogenase to acetaldehyde, which can induce DNA damage. This mechanism does not explain the specific association of alcohol and breast cancer, since alcohol-induced DNA damage should occur in all cell types. To date there are no compelling data to indicate a definitive mechanism for alcohol-induced breast cancer.

In our recent studies, we found that alcohol (ethanol) at physiologically relevant concentrations (≤ blood levels associated with intoxication) causes: 1) down-regulation of the tumor

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suppressor gene *BRCA1* in a dose-dependent manner; 2) significant up-regulation of estrogen receptor (ER)-α expression levels; and 3) a dose-dependent increase up to 10-fold in ER-α transcriptional activity in breast cancer cells.[6] Additionally, we also found that alcohol consumption results in a significant increase in breast cancer cell motility, migration, and invasion, which is associated with decreased expression of the cell-cell adhesion molecules, E-cadherin and α-, β-, and γ-catenins.[6]

Cell motility is an important component of the invasive cancer phenotype. In breast cancer, up-regulation of tumor cell motility and invasiveness is important during the transition from non-invasive cancer (ductal carcinoma in situ) to fully malignant invasive ductal carcinoma. Therefore, these novel findings provide in vitro evidence for a link between alcohol consumption and breast cancer. Based on these new findings, suggested mechanisms underlying the positive association between alcohol consumption and breast cancer risk include the potential influence of alcohol on steroid hormone levels and *BRCA1* as a new inhibitor of ER-α.

ER belongs to the steroid/thyroid nuclear receptor family and is an estrogen-dependent transcriptional factor that regulates growth, development, differentiation, and homeostasis by binding to estrogen response elements in DNA to modulate the transcription of target genes, including progesterone receptors and transforming growth factors, in target organs, such as the breast and uterus. The definitive roles of ER in the development and progression of breast cancer have been elucidated.[7]

Mutations of the breast cancer susceptibility gene *BRCA1* (17q21) confer a high risk for breast and ovarian cancers.[8] *BRCA1* encodes an 1863 amino acid, 220 kDa phosphoprotein with an N-terminal RING finger domain that interacts with cell-cycle proteins, and an acidic C-terminal transcriptional activation domain. *BRCA1* shows multiple biological activities in cell-cycle regulation, apoptosis, and DNA repair and recombination pathways that may be related to its tumor-suppressor functions. Recently, we found that *BRCA1* regulates the transcriptional activity of the estrogen receptor, an important finding that may explain the linkage of *BRCA1* mutations to breast cancer (i.e., loss of the ER-α inhibitory activity due to mutation of *BRCA1* could lead to unopposed estrogenic stimulation of mammary epithelial cells, thus promoting the growth of already initiated mammary epithelial cell clones).[9] The loss of *BRCA1* inhibition of ER-α transcriptional activity also could contribute to sporadic breast cancers, since *BRCA1* mRNA and protein levels frequently are decreased in sporadic invasive breast cancers, as compared with non-invasive cancers and benign tissue.[10]

Conclusion

Our studies link three risk factors—alcohol, ER, and *BRCA1*—to breast cancer development and progression. Our research also provides in vitro data establishing the molecular mechanisms by which alcohol may contribute to breast cancer by two important actions, up-regulation of ER activity and down-regulation of *BRCA1*. These actions form the basis for our proposed working model: alcohol consumption → BRCA1↓ → ER-α activity↑ → breast cancer development and progression. (*Dr. Fan is an Assistant Professor, Albert Einstein College of Medicine, and Chief of the Laboratory of Molecular Oncology, Department of Radiation Oncology, Long Island Jewish Medical Center in New Hyde Park, NY.)* ❖

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Mutations in *SDHD*, a Mitochondrial Complex II Gene, in Pheochromocytomas

By Hartmut PH Neumann, MD, Jörg Schipper, MD, and Charis Eng, MD, PhD, FACP

IT IS BELIEVED THAT APPROXIMATELY 10% OF ALL PHEOchromocytomas are hereditary, i.e., caused by gene mutations which can be transmitted from generation to generation. To date, causes of heritable pheochromocytomas include von Hippel Lindau disease (VHL), which is characterized by germline (in every cell of the body) mutations in the *VHL* tumor-suppressor gene;[1] multiple endocrine neoplasia type 2 (MEN 2), which is characterized by germline mutations in the *RET* proto-oncogene;[2] and rarely, type 1 neurofibromatosis, which is characterized by germline mutations in the *NF1* tumor-suppressor gene.[3,4] Despite advances in human cancer genetics, germline mutations in these genes do not account for all possible hereditary pheochromocytomas.

Hereditary Pheochromocytomas

In mid-2000, Baysal and colleagues identified germline mutations in a presumed tumor-suppressor gene called succinate dehydrogenase subunit d (*SDHD*) in five families that segregated glomus tumors (paragangliomas of the neck).[5] SDHD is the small subunit of cytochrome b (cybS) in the succinate-ubiquinone oxireductase pathway, which comprises mitochondrial complex II. Since glomus tumors are located in the carotid body which has oxygen-sensing capabilities, it made teleological sense that a mutation in a component of a mitochondrial oxidation-reduction pathway was associated with glomus tumors. It should be noted, however, that in these original five families, none had pheochromocytomas. Nonetheless, given the common neural crest origin of paragangliomas and pheochromocytomas, *SDHD* became an excellent novel candidate susceptibility gene for hereditary pheochromocytomas.

A proportion, perhaps one-half, of site-specific familial pheochromocytoma are caused by germline mutations in *VHL*, i.e., are actually cryptic VHL.[6,7] However, the remainder are unaccounted for. Thus, when the five families were examined for the presence of germline *SDHD* mutations, one family that segregated pheochromocytomas and paraganglioma was found to harbor germline *SDHD* mutation.[8]

In a pilot series of 18 unrelated patients with intra-abdominal, catecholamine-secreting pheochromocytomas without VHL, MEN 2, or NF1 by clinical and molecular means or any family history of cancer, two were found to have pathogenic germline *SDHD* mutations, and one was found to have a germline variant of unknown significance.[9] We suspect that the latter germline variant is a pathogenic missense mutation. Thus, the occult germline *SDHD* mutation frequency ranges from 10% to 17% among all apparently sporadic pheochromocytoma cases.

These observations have implications for the patient as well as his/her family. Harboring a germline *SDHD* mutation puts an individual at risk for developing pheochromocytomas, as well as extra-adrenal paragangliomas. If our data can be replicated, then it might be prudent to consider subjecting all pheochromocytoma presentations to germline *SDHD* mutation analysis, because mutation-positive individuals should be subjected to serial surveillance. No gene testing should be performed, however, without the input of clinical cancer geneticists.

Sporadic Pheochromocytomas

While the molecular etiology of heritable pheochromocytoma is at least partially known, the somatic (occurring only in the tumor) genetic etiology of sporadic pheochromocytoma is largely unknown. It is not uncommon that if germline mutation in gene X is found in a syndrome segregating a particular tumor, the sporadic counterpart of that tumor harbors a high frequency of somatic mutations in gene X. For instance, germline mutations of *VHL* cause VHL.

Renal cell carcinoma is an important component tumor in VHL; somatic *VHL* mutations are found in a high frequency of sporadic clear cell renal cell carcinoma.[10] Sporadic pheochromocytomas only rarely have been found to harbor somatic intragenic mutations in *VHL* (< 5% of cases) or in *RET* (< 10% of cases).[10-12] When *SDHD* was examined in sporadic pheochromocytomas, only one of 18 was found to carry a somatic intragenic *SDHD* mutation.[9] Thus, the genetic etiology of sporadic pheochromocytoma remains elusive. Clearly, the pathogenesis of sporadic vs. VHL-related pheochromocytoma is different genetically.

Bender and colleagues examined 17 sporadic pheochromocytoma and 36 VHL-related tumors. They found that while more than 91% of VHL tumors had loss of heterozygosity (LOH) of markers (an indication of the presence of a putative tumor suppressor) on chromosome arm 3p in the region of *VHL*, only 24% of sporadic tumors had LOH in the *VHL* chromosomal region.[13] Instead, LOH of markers on 1p (71%) and 22q (53%) predominated in sporadic pheochromocytomas, as compared to those from VHL cases. Thus, genetic loci that could play a major role in the pathogenesis of sporadic pheochromocytoma could lie on chromosome arms 1p and 22q. Interestingly, MEN 2-related pheochromocytomas may share similar genetic alterations on 1p more akin to sporadic tumors than VHL-related tumors, although these observations were based on relatively small numbers.[14]

Conclusion

Heritable pheochromocytoma can be caused by germline mutations in *RET*, *VHL*, and *SDHD*. *SDHD* is particularly important because it might occur with a frequency as high as 10-17% in apparently sporadic cases of pheochromocytomas. Clinicians must be mindful of this and if in doubt, should refer such individuals for clinical cancer genetics consultation. It also is obvious that other susceptibility genes for hereditary pheochromocytomas do exist and need to be identified. Somatic mutations in *RET*, *VHL*, and *SDHD* account for no more than 15-20% of sporadic pheochromocytomas. Much work is required in this area to elucidate comprehensively the genetic etiology of all sporadic pheochromocytomas. *(Dr. Neumann is an Associate Professor of Internal Medicine, and Dr. Schipper is an Assistant Professor of Otolaryngology, Albert-Ludwigs University, Freiburg, Germany; and Dr. Eng is an Associate Professor of Medicine and Human Cancer Genetics and Director, Clinical Cancer Genetics Program, Ohio State University, Comprehensive Cancer Center in Columbus.)* ❖

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Loss of TAP and LMP Expression in Renal Cell Carcinomas: A Mechanism of Tumor Escape

By Nilanjan Ghosh and Kenneth L. Wright, PhD

THE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I (MHC-I) molecules present endogenous antigenic peptides to the cytotoxic $CD8^+$ T cell population. This interaction between the antigen-presenting cells and the cytotoxic T cells is required for T cell-mediated killing of the infected or transformed cells. Endogenous proteins are degraded to peptide fragments in the cytosol, and peptides with a preferred length of 7-13 amino acids are transported into the endoplasmic reticulum (ER). In the ER or in the *cis*-Golgi, a stable trimolecular complex is formed, composed of a peptide, a newly synthesized MHC-I molecule, and β2-microglobulin (β2m). This complex then is transported to the cell surface. A $CD8^+$ T cell, bearing a T cell receptor (TCR) that specifically recognizes a given MHC-I allele with a uniquely bound peptide, will be stimulated to proliferate and become activated by the interaction. The activated T cells then kill the antigen-presenting cell through the action of perforin and granzymes, thus eliminating the aberrant cells from the body.

TAP1, TAP2, LMP2, and LMP7: Functional Role in the MHC-I Antigen-Presenting Pathway

The proteosome is a multi-catalytic, multi-subunit complex previously demonstrated to be part of the ubiquitin-dependent protein degradation pathway present in all cells. Two proteosome subunits, low molecular mass polypeptide 2 and 7 (LMP2 and LMP7) and a third molecule, MC14 (or LMP9) associate with the 20 s proteosome complex, following induction by the cytokine interferon gamma (IFN-γ).[1,2] Incorporation of LMP2, LMP7, and MC14 into the complex displaces constitutive subunits and modulates the types of peptides generated.[3,4] These proteosomes have been called immunoproteosomes because the specificity of the proteosome complex is altered to enhance the production of peptides cleaved after hydrophobic and basic residues. These peptides often are preferred by MHC-I molecules. Mice deficient in either LMP2 or LMP7 show susceptibility to specific infections due to failure to generate important immunodominant epitopes. Thus, association of LMP2 and LMP7 with the proteosome is important for effective and complete antigen processing and presentation.

The transporters associated with antigen processing (TAP1 and TAP2) are heterodimers that reside in the ER and *cis*-Golgi membranes.[5-7] TAP molecules transport antigenic peptides in an ATP-dependent manner into the ER lumen for binding by the MHC-I heavy chain. TAP physically associates with the MHC-I/β2M dimer through a bridging molecule, tapasin, during peptide loading in the ER.[8] Calnexin and calrericulin are present as molecular chaperones during function of the trimolecular complex. A schematic of MHC-I antigen-presenting pathway is depicted in Figure 1.

Studies on TAP-deficient or mutant cell lines have been invaluable for understanding the importance of TAP in the MHC-I antigen-presenting pathway. Loss of TAP can lead to the absence of MHC-I expression on the cell surface, despite the presence of MHC-I mRNA or a selective inability to present endogenous antigens.[9,10] TAP 1-deficient mice lack MHC-I expression on the cell surface and have severe deficiency in $CD8^+$ T cells.[11]

Tumor Escape

Recognition of the peptide-MHC-1 complex by effector T cells leads to a sequence of events that results in

Figure 1
Schematic of MHC-I Antigen-Presenting Pathway

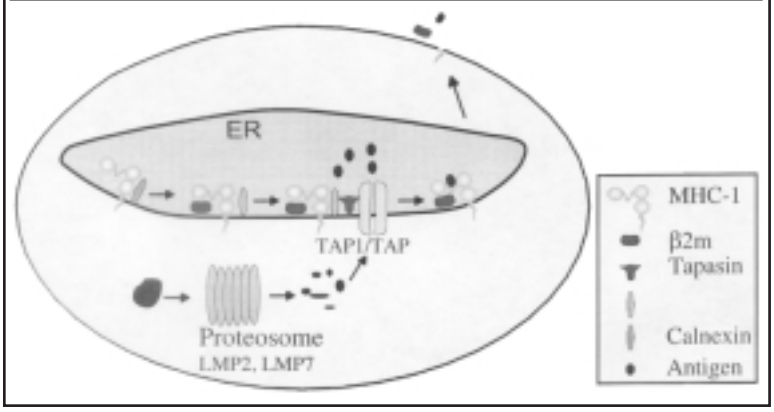
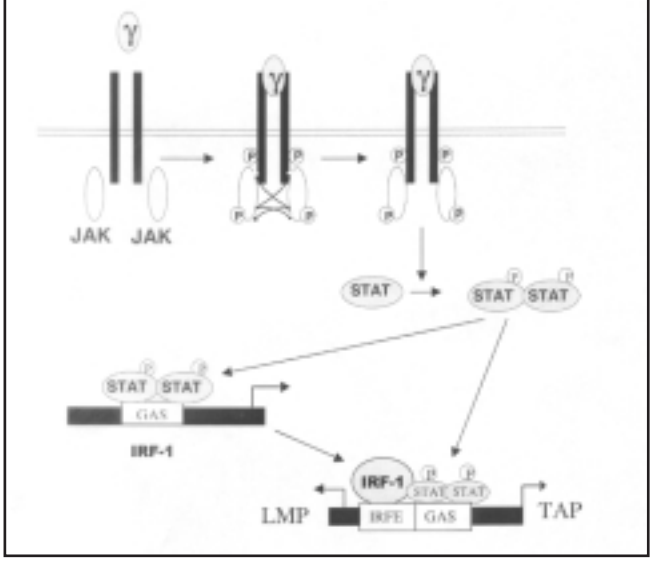


Figure 2

Pathway of IFN-γ Signaling to Gene Activation



lysis of the transformed cell. Thus, T cells can help regulate tumor progression in an immunocompetent host. However, tumor cells have developed mechanisms to escape recognition from the patrolling cytotoxic T cells. This escape from immune surveillance may lead to uninhibited proliferation of the transformed cells, despite the presence of tumor-reactive T cells. One direct mechanism of tumor escape is to prevent expression of MHC-I molecules with tumor-associated peptides on the cell surface. This could be accomplished by inhibiting the expression or directly mutating the MHC-I, TAP, or LMP molecules. In addition, since each of these genes is up-regulated by interferon-gamma (IFN-γ), inhibiting the IFN-γ signaling pathway would significantly inhibit antigen presentation. This ultimately may render the tumor cells resistant to lysis by MHC-I antigen-restricted cytotoxic T cells and allow tumor progression.

Transcriptional Regulation of *TAP* and *LMP* Genes

All of the *TAP1*, *TAP2*, *LMP2*, and *LMP7* genes are adjacent to each other and located within the MHC locus on human chromosome 6. The *TAP1* and *LMP2* genes are transcribed from a shared bidirectional promoter. A minimal 593 bp region separating the ATG translation initiation codons of both these genes is sufficient for concurrent expression in both directions.[12] The absence of TATA boxes is well correlated with the presence of multiple start sites for both these genes. The transcriptional regulation of these genes is mediated by the *cis*-acting elements in the promoter region proximal to the *TAP1* gene. They include an interferon response element and a gamma-activated sequence (IRF-E/GAS) composite site that is bound both by the interferon response factor-1 (IRF-1) and the signal transducer and activator of transcription 1 alpha (STAT1α). Both IRF-1 and STAT1 activity are stimulated by IFN-γ treatment. Recently, it also has been demonstrated that unphosphorylated STAT1 and IRF-1 can form a complex and bind to the promoter region to mediate constitutive LMP2 transcription.[13] The TAP1/LMP2 promoter also utilizes an SP1 binding site for constitutive activity and a NFκB site, which can respond to multiple stimuli, including TNF-α.

INF-γ Signaling Pathway

The pathway of IFN-γ signaling to gene activation has been elegantly deciphered in the last several years *(see Figure 2).* IFN-γ, a cytokine secreted by T cells and natural killer cells, has antiproliferative effects and induces *MHC-I*, *TAP1*, *TAP2*, *LMP2*, and *LMP7* genes. IFN-γ treatment of certain renal carcinoma cell (RCC) lines leads to induction of *TAP1* and *LMP2* genes via the IRF-E/GAS DNA element proximal to the TAP1 promoter, and mutation of this element abolishes the inducibility. This TAP and LMP induction, which is independent of new protein synthesis, is followed by MHC-I expression.[14] This may be responsible for enhanced recognition of RCC by the immune system.

The binding of IFN-γ to its receptor leads to activation and phosphorylation of the receptor-associated Janus kinases (Jak1 and Jak2). Receptor tyrosine phosphorylation generates a binding site for STAT1α, which is subsequently phosphorylated by Jak1 and Jak2 to form active STAT1α homodimers. Activated STAT1α translocates to the nucleus where it binds to the DNA at GAS elements in the promoters of many IFN-γ-induced genes including the *IRF-1* gene promoter. IRF-1 expression is induced subsequently.[15] The IRF-1 protein binds to IRF-E DNA elements in additional IFN-γ-induced genes. The TAP1/LMP2 promoter utilizes both direct induction by STAT1α and secondary induction by IRF-1 at its composite IRF-E/GAS DNA element. Induction of TAP and LMP by IFN-γ could assist in overcoming the expression of unstable MHC-I molecules found in multiple tumor types, including several RCC lines.[9,10,14] Thus, restoring the antigen-presenting machinery by inducing TAP and LMP may improve the cytotoxic T cell-mediated, antigen-specific antitumor effect in patients bearing carcinomas with TAP and LMP deficiency.

Inability of IFN-γ to Induce TAP1 and LMP2 in Renal Carcinoma Cell Line Caki-2

Recently it has been shown that RCC cell lines Caki-1 and Caki-2 show low constitutive levels of TAP1 and LMP2.[10] On IFN-γ treatment, TAP1 and LMP2 were

induced only in Caki-1 cells, but not in Caki-2. IFN-γ resulted in a two- to three-fold induction of *TAP1* and *LMP2* genes in Caki-1 cells, but had no effect in Caki-2 cells.[16] Consistent with previous results, IFN-γ was unable to induce TAP and LMP expression in either cell line when the IRF-E site was mutated. Because STAT1 and IRF-1 have been shown to be involved in the transcriptional regulation of *TAP1* and *LMP2* genes, we investigated whether the loss of TAP1 and LMP2 expression in the RCC cell line, Caki-2, was attributable to deficiencies of these factors.[16]

IRF-1 Is Not Bound to the IRF-E Site in Caki-2 Cells

In vivo genomic footprinting is an excellent technique for examining DNA-protein interactions within the cell. Briefly, live cells are treated with dimethyl sulfate (DMS), which permeates through the membranes into the nucleus and methylates the guanine residues in DNA. However, if a protein is bound to DNA, the guanine residue can be protected from methylation. Then the DNA is isolated from the cells and treated with piperidine, which cleaves at the methylated guanine residues, leading to formation of a DNA ladder. The gene of interest is amplified by PCR and analyzed on a sequencing gel. The results are compared to deproteinated DNA from the same cells, which was isolated prior to treatment by DMS. By using this technique we determined that the Caki-1 cells showed a strong protection of guanine residues upon IFN-γ induction at the IRF-E site in the bidirectional promoter region, indicating that this site was occupied in vivo. However, in Caki-2 cells this site was not occupied upon IFN-γ induction.

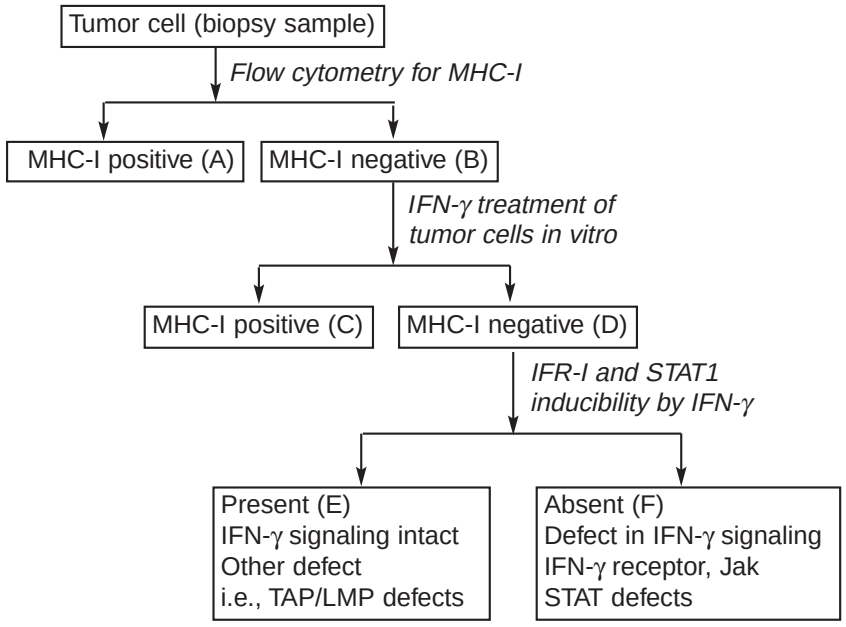
To determine the identity of the proteins bound to the promoter region in these cells, we looked at in vitro DNA protein-binding ability by electrophoretic mobility shift assays. A radio-labeled oligonucleotide containing the IRF-E element from the TAP1/LMP2 promoter was incubated with nuclear extracts from Caki-1 and Caki-2 cells. After five hours of IFN-γ treatment, protein bound to the IRF-E site was easily detected in the Caki-1 cells. However IFN-γ treatment failed to induce IRF-1 DNA binding activity in Caki-2 cells.

Mechanism of IRF-1 Down-Regulation in Caki-2 Cells

Activation and phosphorylation of STAT1 is required for IRF-1 induction by IFN-γ. Since activated STATs form homodimers and bind to DNA, this phenomenon can be used as a tool for determining the presence of activated STAT proteins in the cell. No activated STAT1 protein DNA binding activity was detectable in Caki-2 cells as opposed to Caki-1 cells. Activation of STAT1 also can be measured directly by using antibodies that specifically recognize the phosphorylated form of STAT1. Upon IFN-γ treatment, phosphorylated STAT1 is induced in Caki-1 cells, but not in Caki-2 cells. However, constitutive levels of unphosphorylated STAT1 are detectable in both Caki-1 and Caki-2 cells. This indicates that the defect in Caki-2 cells lies not in the expression of STAT1, but rather in the activation of STAT1. Tyrosine phosphorylation of Jak1 and Jak2 induces STAT1 activation via the IFN-γ signaling pathway. Induction of Jak1 and Jak2 phosphorylation is seen in Caki-1 cells, but not in Caki-2 cells. However, constitutive levels of Jak1 and Jak2 proteins are detectable in both cell lines. There are two possibilities to explain this phenomenon. Either there is a mutation in the Jak1 and/or Jak2 proteins which prevents them from being activated, or there is a mutation in the IFN-γ receptor

Figure 3

Tumor Screening Schematic



Patients belonging to group C and E could be treated with IFN-γ early in the disease. In groups E and F, IFN-γ would be effective only if it is supplemented by correction of other defects by gene therapy.

rendering the Caki-2 cell line unresponsive to IFN-γ. Overexpression of Jak1 and/or Jak2 was not able to re-establish inducibility of the Caki-2 cell line to IFN-γ. Thus, the defect in this cell line is not due to abnormal Jak proteins. Although IFN-γ receptor is expressed in the Caki-2 cell line, it appears that mutations in the receptor hamper the IFN-γ signaling pathway in these cells.

Role of IFN-γ as an Immunotherapeutic Agent in Renal Cell Carcinoma

Patients with defective IFN-γ signaling are susceptible to severe disseminated infections.[17,18] Importantly, IFN-γ sensitivity is required for the enhancement of tumor immunogenicity and elicitation of an effective tumor-specific immune response. A large proportion of tumors show IFN-γ unresponsiveness,[19] and these tumors may be able to circumvent detection by the host immune system. RCC, an epithelial cell tumor, is the most common malignancy of the kidney. The incidence has been slowly rising and it is estimated that in the United States, 31,200 people will be diagnosed with kidney cancer in the year 2000, and 11,900 will die of the disease.[20] At diagnosis, 55% of patients have either metastatic disease or locally advanced tumors with lymph node and/or local organ involvement.[21] Patients with metastatic disease have a three- to five-year survival rate of less than 5%.[22]

The rationale for using immunotherapy for treating advanced and metastatic RCC came from the observation that many of these tumors demonstrated infiltration by lymphocytes and macrophages.[23] In 25% of patients, these tumor-infiltrating lymphocytes were able to lyse autologous tumor cells in a MHC-restricted manner and/or produce IFN-γ in response to autologous tumor but not allogenic RCC.[24] A group of RCC is relatively responsive to cytokines with growth inhibitory and immunomodulating properties, such as IFN-α and IFN-γ. Low-dose IFN-γ treatment was effective in maintaining long-term complete remissions in approximately 15% of RCC patients with limited disease.[25-27] The up-regulation of TAP and LMP molecules by IFN-γ leading to an effective MHC-I processing machinery may be one mechanism by which low-dose IFN-γ therapy has its effect.

This hypothesis is further supported by the demonstration that transfer of *TAP1* gene in a RCC cell line enhanced its immunogenicity.[28] However, the observations that some RCCs do not up-regulate TAP and LMP in response to IFN-γ and our findings that the IFN-γ signaling pathway can be defective in these tumors, indicate that examining the IFN-γ response in RCC patient samples could be informative to estimate the likely success of low-dose IFN-γ.

Conclusion

Studies of TAP1 and LMP2 transcriptional regulation in tumor cells have provided us essential information for designing genetic and immunotherapeutic strategies to enhance MHC-I antigen presentation to combat cancer. One such strategy would be to screen tumor samples obtained during biopsy or surgery in RCC patients to design specific therapeutic measures. A simple schematic is described in Figure 3. *(Mr. Ghosh is a PhD Candidate, Department of Biochemistry and Molecular Biology, Interdisciplinary Oncology Program, College of Medicine; Dr. Wright is an Assistant Professor, Interdisciplinary Oncology Program, and Member in Residence, H. Lee Moffitt Cancer Center, University of South Florida in Tampa.)* ❖

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The *NUP98* Gene in Human Hematological Malignancies

By Harish G. Ahuja, MD

NON-RANDOM CHROMOSOMAL TRANSLOCATIONS SUCH as translocations, deletions, and inversions frequently are associated with a wide variety of hematological malignancies and generally are thought to be causal events in the process of leukemic transformation.[1] In most instances, chromosomal translocations fuse sequences of a transcription factor, transcription modulator, or receptor tyrosine kinase to those of a normally unrelated gene, resulting in a chimeric protein with oncogenic properties.[1]

Repositioning of transcriptional control genes in the vicinity of highly active promoter/enhancer elements, such as those associated with the immunoglobulin or T-cell receptor genes, is a second mechanism by which translocations induce malignancy.[1] Similar chromosomal translocations also are associated with therapy-related myelodysplastic syndromes and/or acute myelogenous leukemia (t-MDS/AML) and are thought to arise as a result of exposure to various forms of chemotherapy.[2] Most cases of t-MDS/AML arising after exposure to alkylating agents are associated with loss of chromosomes 5 and/or 7 or various parts of the long arm of these chromosomes.[2] On the other hand, agents that target the enzyme topoisomerase II (topII), such as epipodophyllotoxins or anthracyclines, cause leukemia characterized by a short latency period, monocytoid features, and usually balanced translocations involving the *MLL* gene on chromosome 11q23.[3,4] Other balanced translocations, e.g., t(8;21), t(15;17), and inv(16), which

characteristically occur in de novo AML have been reported, albeit infrequently, in t-MDS/AML as well.[4-6]

The *NUP98* Gene

The *NUP98* gene on chromosome 11p15 is a 98 KD component of the nuclear pore complex that is presumed to function as a docking protein controlling nucleocytoplasmic transport.[7] This docking function is mediated by multiple FXFG repeats located in the N-terminal portion of the gene.[7] Several recent reports have indicated that the *NUP98* gene is found at the breakpoints of several distinct chromosomal breakpoints in human hematological malignancies. These include: t(7;11)(p15;p15), t(2;11)(q31;p15), t(1;11)(q23;p15), inv(16)(p15q22), and t(4;11)(q21;p15). Chimeric mRNAs spliced across the breakpoints fuse the FXFG repeats of NUP98 with HOX A9, HOX D13, PMX-1 (all homeodomain-containing proteins), DDX 10 (a putative RNA helicase), and RAP1GDS1 (a guanine nucleotide exchange factor).[8-13] Significantly, with the exception of the t(4;11) which has been reported in a patient with T cell acute lymphoblastic leukemia, all of the aforementioned translocations have been reported in patients with t-MDS/AML, arising in the context of exposure to multi-agent chemotherapy that has included a topII inhibitor.

The t(11;20)(p15;q11.2) is a rare but recurrent chromosomal translocation that has been reported in patients with MDS, AML, and polycythemia rubra vera.[14-16] We identified two children who developed t-MDS/AML associated with the t(11;20) following exposure to multi-agent chemotherapy that included a topII poison.[14] Using 3' RACE we have cloned the fusion transcript arising as a result of this translocation and have demonstrated an in-frame fusion between 5' NUP98 FXFG repeats and the main body of DNA topoisomerase 1 (topI).[17] TopI normally catalyses a series of transesterification reactions during which it generates transient single-stranded DNA breaks that result in topological transformations in DNA.[18] TopI also has been shown to be involved in DNA replication, transcription, and recombination, as well as chromosome condensation.[18] The topI protein can be organized into four distinct domains: a C-terminal catalytic region that contains the active tyrosine; a non-conserved linker domain; a conserved "core" domain; and a N-terminal region that contains a nuclear localization signal.[18] Although it has been shown that this N-terminal region is dispensable for catalytic activity, several potentially significant interactions involving this region of topI have been reported. These include interactions with nucleolin, SV40 large T antigen, and the SF2/ASF splicing factor.[19-21] The NUP98-topI fusion protein lacks this N-terminal region and it is possible that loss of regulatory functions dependent on this N-terminal region may contribute to leukemogenesis. It also has been recently been shown that topI interacts with p53.[22] It is conceivable that loss of this interaction as a result of the translocation also could contribute to leukemogenesis. Finally, it has been demonstrated that the NUP98-HOX A9 fusion protein can transform NIH 3T3 fibroblasts in vitro.[23] The mechanism suggested is one in which HOX A9-responsive genes are activated by the transactivating properties of the NUP98 FXFG repeats. A similar mechanism could explain the potential leukemogenicity of the NUP98-topI fusion.

To investigate potential mechanisms responsible for the generation of the t(11;20) associated with t-MDS/AML, we cloned and sequenced the genomic breakpoints from the two patient samples.[24] Nucleotide sequence analysis of the germline as well as rearranged alleles revealed almost perfectly balanced translocations with no net gain or loss of DNA. We did not detect any known recombinogenic sequences such as Alu repeats, purine/pyramidine repeat regions, palindromi sequences, consensus x-like sequences, heptamer-nonamer sequences, or putative topII consensus recognition sites at or near the breakpoints. However, close analysis of the breakpoint junction sequences revealed four nucleotide microduplications at the breakpoint junctions. The simplest hypothesis to explain the four nucleotide microduplications at the breakpoint junctions proposes that the translocations were generated by four nucleotide staggered double-stranded DNA breaks, allowing the four nucleotide overhang to serve as a template on each derivative chromosome. Given the history of exposure to topII poisons and the fact that such drugs stabilize the staggered breaks when topII binds covalently to DNA, it seems plausible that topII acted as a catalyst in inducing these rearrangements perhaps through an exchange of topII subunits. Taken together with previous reports of *NUP98* rearrangements in patients with t-MDS/AML, our findings suggest that *NUP98*, like *MLL*, is a frequent target for chromosomal translocations in patients with t-MDS/AML that develops after exposure to multi-agent chemotherapy containing a topII poison.

NUP98 rearrangements are not restricted to patients with t-MDS/AML and occur in patients with de novo AML as well. We recently have reported the cloning of a t(9;11)(p22;p15) from a patient with de novo AML and have demonstrated that the translocation results in a fusion between the *NUP98* gene and the gene encoding the transcriptional co-activators p52 and p75, both of which are derived through alternative splicing from a single gene known as lens epithelium-derived growth factor (*LEDGF*).[25] Both NUP98-p52 and NUP98-p75

chimeric mRNAs were detectable in the patient sample. The p52 and p75 proteins are homologous to the hepatoma-derived growth factor (HDGF) and the HDGF-related proteins 1 and 2 (HRP-1 and HRP-2).[26] The highest degree of homology (80%) is found in the N-terminal amino acid residues also known as the HATH region (homologous to the N-terminus of HDGF) and it is this region that is lost from the fusion protein. The C-terminal region shows similarity to HMG-1, a multifunctional non-histone protein involved in many steps of gene regulation. p52 is a potent transcriptional co-activator and is thought to mediate functional interactions between upstream sequence-specific activators and the general transcriptional apparatus. It also has been shown to interact with the essential splicing factor ASF/SF2 to modulate mRNA splicing.[26] p75 is a less potent co-activator than p52 and does not interact functionally with ASF/SF2. However, it has been shown to function as a growth and survival factor for lens epithelial cells, keratinocytes, and skin fibroblasts.[27]

Conclusion

It is intriguing that two fusion proteins with potentially differing functions were detectable. The relative contribution of each of these fusion proteins to leukemogenesis is under study.

The exact mechanism whereby these NUP98 fusions cause leukemia remains speculative. One possible scenario is the disruption of functions normally attributable to *NUP98* or the various partner genes. Another scenario is through a "gain of function" acquired as a result of fusion to the NUP98 FXFG repeats. Future experiments in which *NUP98* or the various partner genes are mutated and homozygously inactivated in "knock-out" mice or transgenic mouse models in which the various fusion genes are overexpressed will undoubtedly shed light into some of these mechanisms. *(Dr. Ahuja is a Medical Oncologist at the University of Wisconsin Comprehensive Cancer Center in Wausau.)* ❖

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Funding News

Human Frontier Science Program

The Human Frontier Science Program (HFSP) supports program grant funding for molecular approaches to biologic research and aspects of neuroscience. Emphasis is based on novel collaborations that bring together scientists from different disciplines to focus on various aspects of life sciences research. Collaborative programs involving such disciplines as chemistry, physics, mathematics, computer science, and engineering are encouraged. Program grant applications may be made for up to $500,000 for the entire term per year based on approximately $100,000 per team member plus funds for special equipment needed for the collaboration. More information is available at the HFSP Web site: http://www.hfsp.org. Primary applicants are expected to register and submit a letter of intent electronically at the Web site. Invitations then will be sent out to a limited number of applicants for the full application.

The HFSP also offers funding to support the collaborative research efforts of scientists early in their careers. All team members should be within five years of obtaining an independent position and within 10 years of obtaining their PhD. Grants will be made in the amount of $250,000 per team per year. The young investigators should have completed one or two periods of postdoctoral training and be in a position that allows them to initiate and direct their own independent line of research. The deadline for letter of intent is March 30, 2001. ❖

CME Questions

10. The correlation between breast cancer susceptibility and alcohol consumption:
a. is unrelated to amount of alcohol consumed.
b. appears to be enhanced by postmenopausal estrogen replacement therapy.
c. is supported by an increase in cell adhesion molecule expression.
d. is supported by an up-regulation of *BRCA1*.

11. Succinate dehydrogenase subunit D is the small subunit of:
a. cytochrome b.
b. lysozyme.
c. topoisomerase I.
d. catalase.

12. The transporters associated with antigen processing (TAP1 and TAP2):
a. are homodimers.
b. reside in mitochondria.
c. transport antigenic peptides into the ER lumen for binding by the MHC-I heavy chain.
d. require cGMP for activity.

13. Most cases of t-MDS/AML arising after exposure to alkylating agents are associated with loss of all or part of chromosomes:
a. 3 and/or 6.
b. 4 and/or 9.
c. 5 and/or 7.
d. 8 and/or X.