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Description and validation of high-throughput simultaneous genotyping and mutation scanning by high-resolution melting curve analysis

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A key step in the search for potentially deleterious genetic variants in known or candidate susceptibility genes is mutation screening of the exonic/coding sequences and splice sites of the entire gene in large subject series. Mutation scanning using high-resolution melting curve analysis has been described as an effective and sensitive method to detect sequence variations. However, the presence in a mutation screening amplicon of a common SNP may considerably complicate the interpretation of results and increase the number of samples flagged for sequencing by interfering with the clustering of melt curve groups according to melting profiles. A protocol describing simultaneous high-resolution gene scanning and genotyping has been reported. We aimed to validate the efficiency of this method in a large-scale case-control mutation study. The method is based on hybridization of unlabeled oligonucleotide probes designed to anneal to the common polymorphic site and blocked at the 3' end to prevent extension during PCR amplification. A single asymmetric PCR in presence of both LCGreen® Plus dye and unlabeled oligonucleotides probes leads to simultaneous production of probe-target and whole amplicon double-stranded DNA duplexes that can be analyzed from the same HR melting run. Common SNP genotypes are called from the probe-target melting data. Analysis of the whole amplicon melt curves (e.g., mutation scanning) is then performed separately on heterozygous and homozygous samples to distinguish curve shape differences due to other unknown variants. To validate this approach, we chose the 22nd and 36th coding exons of the ATM gene (NM_000051), both of which contain a common SNP that interferes with standard HR-melt mutation scanning. The assay was performed on 650 cases and 650 controls enrolled in an international breast cancer genetics study. The exon 22 amplicon contains a common 1bp deletion. The exon 36 amplicon is more challenging, as it contains a common SNP adjacent to an uncommon SNP. In both cases, we succeeded in identifying rare known and unknown variants while dramatically reducing the amount of sequencing required. In presence of common SNPs, combining genotyping and mutation scanning showed good accuracy for the identification of rare variants. This simple procedure greatly reduces the number of samples to sequence. Hence, high-resolution melting analysis is a rapid, efficient and cost-effective tool that can be used for high-throughput case-control mutation screening for research, as well as for molecular diagnostic and clinical purposes.

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Natural lgl-tumor supressor alleles in Drosophila: genetics and epigenetics of their stress-adaptive effects

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Drosophila tumor suppressor (TS) gene lethal (2) giant larvae (lgl) is the first case of monogenically controlled neoplasia found in animals (Gateff, 1978). The loss of lgl function leads to overgrowth of imaginal discs (benign tumor) and invasive malignant brain neuroblastoma. The Lgl protein turned out a new type of TS involved in signal transduction, asymmetric cell division and tissue architecture. lgl orthologues have been discovered in diverse species. Mutations of TS human homologue Hugl were found in about 70% of solid carcinoma. Surprisingly, our long-termed studies showed omnipresence of loss-of-function lgl alleles in heterozygotes in distant Drosophila populations. Most of natural lgl alleles appeared to be deletions. To address this population paradox we analyzed some parameters of biological adaptation of lgl loss-of-function heterozygous carriers both in normal and stress conditions. The summarized data include: (i) embryonic survival and aging dynamics in normal and soft temperature stress conditions, (ii) resistance to RNA lethal infectious DCV-virus, (iii) maternal and transgeneration effects of the one dose of lgl TS loss on the progeny survival and life span. Animals heterozygous on 15 natural lgl-alleles showed increased resistance to infectious highly virulent DCV virus. We demonstrated significant improvement in survival and aging for one dose of the TS-deficient animals namely in the temperature stress conditions. The effect was maternal and sex-dependent. Impulse thermal stress (3 hours, 29°C) applied during five successive stage of lgl/+ mothers oogenesis showed essential F1 pre-zygotic survival and longevity increase. The primary germ cells differentiation stage was epigenetically most stress sensitive. Observed stress-adaptive effects of natural widespread lgl alleles are discussed in the frame of phenomenon of possible haploadaptivity and diplo-redundancy of some vital genes. The data obtained are important for an understanding of population spreading of some risk factor mutations in humans and their health effects in environment/stress conditions. The data draw attention to the possible oogenesis-dependent transgeneration aspect of determination and expression of human mutant factors. They model pre-zygotic transgeneration epigenetic effects of TS deletion heterozygozity on aging and longevity, some multiple epidemic pathologies.

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Cis-acting genomic elements of Pas1 locus control Kras mutability in lung tumors

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Inbred mouse models display different susceptibilities to spontaneous or carcinogen-induced lung cancer. Genetic linkage analyses have uncovered a number of Quantitative Trait Loci (QTLs) modulating lung cancer risk and development in mice. In particular, Pas1 (Pulmonary Adenoma Susceptibility 1) locus, located in the distal region of chromosome 6, appears to play a major role in the inherited predisposition to spontaneous and chemically induced lung tumorigenesis in mouse models. Pas1 locus displays two different haplotype: a susceptible one (A/J-like) and a resistant one (C57BL/6J-like). Many reports suggested that Kras, one of the six gene mapping in Pas1 core haplotype, is a good candidate for Pas1 locus function. In order to investigate Kras role in lung tumorigenesis, we propose a new mouse model developed by replacing Kras gene with Hras transcript in a Pas1 susceptible background and crossing these mice with either a resistant (C57BL/6J) and a susceptible (A/J) strain. After lung cancer induction with urethane treatment, we observed that, in both crosses, heterozygous mice carrying the Hras-replacement gene were more prone to develop lung tumors than wide-type mice, indicating that Hras-replacement gene not only supplies Kras functions but it is also more active. Furthermore, most of the lung tumors carried a Gli61Leu substitution in Hras-replacement gene, whereas no mutations were observed in the endogenous Hras gene. Thus we suggest that Pas1 locus context is able to drive ras genes mutability. Moreover, in tumors obtained from mice carrying Hras-replacement gene, the mutation frequency affecting the wild-type Kras gene was higher when this gene was located in the susceptible (A/J) than in the resistant (C57BL/6J) Pas1 locus context (12% versus 0%, -log P=5.0). These findings indicate that cis-acting elements located in Pas1 locus are the functional components modulating Kras gene mutability and controlling susceptibility to lung tumorigenesis in mouse strains.

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Differential expression of Trefoil factor family reflects their different roles in liver fluke related Cholangiocarcinoma

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Background: The Trefoil factor (TFF) gene family composes of TFF1, TFF2 and TFF3. TFF plays an important role in restitution and repair of the epithelium in response to mucosal injury. However, TFF peptides are overexpressed in several human solid tumors. Prolonged inflammatory caused by parasitic infection frequently occurs in liver fluke related cholangiocarcinoma (CCA), TFF may be constitutively activated for repairing and subsequently undesirable effects during chronic inflammation may lead to tumor development and progression. This study attempted to explore TFF genes from genomic through mRNA and protein expression and clarify correlation between DNA copy number and mRNA and protein expression of TFF genes in CCA patients. The associations between TFF gene expression and clinicopathological parameters were also determined by statistical analysis.

METHODS: Tumor tissues from 110 CCA patients were used to determine DNA copy number, mRNA and protein expression of TFF genes. Correlations between DNA copy number and mRNA expression of TFFs were analyzed by linear regression. Association between mRNA and protein expression, TFF expression and clinicopathological parameters of CCA patients were evaluated by means of the Chi-square test.

RESULTS: No significant relationship between DNA copy number and mRNA expression of TFF genes was found. In normal bile ducts, TFF

mRNAs showed low expression and correlated with protein expression. In tumor cells, mRNAs of TFF1 and TFF3 were found increasingly compared to normal tissues (P = 0.008, P = 0.067, respectively). Protein expressions of TFF1, TFF2, and TFF3 in tumor tissues were positive staining at 50.9%, 25.5%, and 49.1%, respectively. The significant association between mRNA and protein was found only in TFF3 (P = 0.028). TFF3 protein expressed widely in normal bile ducts and markedly increased in pre-malignant and tumor cells. Increased expression of TFF1 protein was found in pre-malignant and tumor tissues. TFF2 protein rarely expressed in normal bile ducts and pre-malignant cells but increasingly expressed in tumor. Furthermore, TFF1 and TFF2 proteins were associated with nerve invasion.

CONCLUSIONS: No significant associations between DNA copy number, mRNA expression and clinicopathological data as well as survival time were found. Our studies suggest that TFF3 may be the first and main TFF which plays beneficial effect in cytoprotective bile duct system, whereas TFF1 may play a role as an initiator of CCA development at the pre-malignant stage and promote tumor progression at tumor stage. The alternative expression of TFF2 may play a crucial role in the enhancement of tumor progression in CCA patients.

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Steroid metabolism gene CYP17, CYP1A1*2B, CYP1A1*2C and risk of breast cancer in mexican women

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Background. Breast cancer is the 2nd cause of women mortality in Mexico, increasing this rate over the past ten years. Functional polymorphisms in genes encoding steroid metabolizing enzymes may contribute to this understanding by serving as surrogate markers for altered long-term hormone exposure and, thus, as biomarkers of individual breast cancer susceptibility. In order to determine the impact of CYP17 and CYP1A1 genotypes on the risk to develop breast cancer, we realized a case-control study of breast cancer patients and healthy controls among women invited to participate in this study from a Public Hospital in Mexico City.

Methods. 90 breast cancer patients and 87 healthy contol, who had given their informed consent were included. All breast cancer patiens had pathologically confirmed as breast carcinoma, all were diagnosed and treated at the "1o de octubre" hospital. Epidemiological questionnaire and genotyping data were obtained. CYP17 and CYP1A1 were genotyped using PCR/restriction fragment lenght polymorphism. For CYP17 a single nucleotide polymorphism at the 5`untraslated region of the CYP 17 was done (MspA1 restriction sitie). Two polymorphism for CYP1A1 were analized: 2455 A>G (CYP1A1*2B) and 4889 A >G (CYP1A1 *2C).

Results. We found an increased risk of breast cancer in women carrying the allele CYP1A1*2B. The odds ratio was 2.6 (CI95%= 1.08-6.4; p <0.032). In the stratified analysis, this risk was increased when CYP1A1*2B and CYP1A1*2C were presented together. The odds ratio was 3.4 (CI95%= 1.2-9.3; p<0.017). Regarding the CYP17 genotype, it was not preliminary associated with breast cancer risk. Conclusions. These results suggest that CYP1A1*2B and CYP1A1*2C genotype may be a biomarker for breast cancer risk in our general Mexican population.

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p53 gene alterations and HPV16 infection in early stages of cervical carcinomas in Serbia

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Background: Cervical cancer is the second most common malignant disease among women. Incidence rate (age standardized) of cervical carcinoma in Serbia is the highest in Europe. p53 is mainly inactivated at protein level in carcinomas associated with human papilloma virus (HPV) infection, such as cervical carcinomas. These tumors contain low rate of p53 mutations. It isn't clear if p53 mutations additionally confer impact to prognosis of disease. The role of polymorphic variant at codon 72 of p53 gene as a risk factor for cervical cancer development and patient's prognosis is controversial. The aim of study was to determine the frequency of p53 mutations and polymorphic variants of codon 72 among cervical carcinomas.

Patients and methods: 53 cervical carcinomas patients, FIGO stage I (50) and II (3) were included in study. The majority of cases (n=49) were squamous cell carcinoma. 30/32 patients who received adjuvant radiotherapy were followed-up. DNA was isolated by salting out method from tumor tissue (n=53) and blood (42/53). Mutations in exons 4 to 8 of p53 gene were detected by PCR-SSCP (polymerase chain reaction- single-stranded conformational polymorphism) electrophoresis and confirmed by direct sequencing. HPV16 was examined in 51/53 tumor by PCR-PAGE (polyacrilamide-gel) electrophoresis. Codon 72 polymorphism was assessed by RFLP (restriction fragment-length polymorphism) method.

Results: Five p53 mutations were detected in 4/53 patients in FIGO stage I squamous cell carcinoma (one patient had double mutations). 25/42 patients exhibited Arg/Arg genotype of 72 codon polymorphism. 3/5 p53 mutations were associated with Arg/Arg and 2/5 with Arg/Pro genotype. There was no statistically significant difference in the number of relapses among patients with different genotypes at codon 72. HPV16 type was detected in 29/51 cervical carcinomas. Statistically significant difference in the frequency of Arg/Arg genotype between HPV-16 positive and HPV16 negative patients was not observed. Relapse of disease occurred only in two patients - both with Arg/Arg genotype and HPV16 positive. One of them exhibits p53 mutation.

Conclusion: Results showed low incidence of p53 mutations and prevalence of Arg/Arg genotype polymorphic variant of codon 72 of p53 gene in early stages of cervical carcinomas in Serbia.

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Genetic polymorphism of CYP1A1 & CYP2D6 in Indian chronic myeloid leukemia patients

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BACKGROUND: Chronic myeloid leukemia (CML) is a clonal hematopoetic stem cell disorder. Genetic polymorphism of genes encoding carcinogen-metabolizing enzymes, namely, phase I cytochromes P-450 (CYPs) have been shown to influence the risk to develop cancer. It has been suggested that individuals possessing a modified ability to metabolize carcinogens are at increased risk of cancer. The cytochrome P450 (Phase–I) system is involved in the metabolism of both endogenous and exogenous molecules, CYP1A1 and CYP2D6 are two such genes which effect individual susceptibility towards the risk for cancer from environmental agents. Hence the present study was designed to find out the allelic frequency of CYP1A1 gene (*2A,*2B,*4 alleles) and CYP2D6 gene (*4 allele) in North Indian CML patients and racially matched controls.

AIM: Owing to the importance of CYP1A1 and CYP2D6 genetic polymorphism as risk factor in various cancers, the study was aimed to detect the prevalence of their genetic polymorphism in North Indian CML patients and racially matched controls. Further, it will help to determine the association of these allelic variants if any, as risk factor to develop CML.

METHODS: DNA isolation was carried out by standard proteinase K and phenol chloroform method. The prevalence of CYP1A1 *2A,*2B,*4 alleles and CYP2D6*4 allele was carried out by PCR-RFLP method (Krajinovic et al, 1999). PCR products were separated using 2% agarose gel. The relationship between these alleles and risk of CML was assessed by means of chi square test. The odds ratio (OR) with 95% confidence limits was calculated by logistic regression.

RESULTS: CYP1A1 mutations T6235C (m1), A4889G (m2) and C4887A (m4) were characterized by PCR-RFLP. These mutations were then used to define 3 distinct alleles, CYP1A1 *2A (presence of m1 only), *2B (both m1 and m2) and *4 (m4 only). The frequencies of CYP1A1 alleles *2A *2B and *4 in cases were 21.8% (12/55), 18.1% (10/55), and 9% (5/55), respectively. The allelic frequencies of CYP1A1 genes (*2A,*2B and *4(in controls were 32% (24/75), 16% (12/75), and 4% (3/75), respectively.

The allelic frequencies of CYP2D6*4 in CML patients of homozygous wild, heterozygous and homozygous mutant alleles, were 77% (35/45), 13% (6/45) and 8% (4/45). In controls these frequencies were 75% (42/56), 14% (8/56) and 10% (6/56) respectively.

CONCLUSIONS: A higher frequency of CYP1A1*2A was observed in controls as compared to CML patients. Thus the study provides an evidenced based data, which indicates a reduced risk for CML in individuals carrying the mutant allele CYP1A1*2A.

The results did not support the hypothesis that mutant alleles of CYP2D6*4 gene which are actively involved in activation of carcinogens, will be at greater risk to develop CML. Our attempt to study role of CYP2D6*4 allelic variants in CML is promising, however, study needs to be taken further with larger sample size to validate the results.