SUSCEPTIBILITY MARKERS IN NORMAL SUBJECTS: A PILOT STUDY FOR THE INVESTIGATION OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN RELATED DISEASES

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While 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is one of the most potent carcinogens known in rodents[1], direct evidence of carcinogen effect on humans is equivocal. To date the biological reasons for a marked difference in sensitivity between humans and rodents are not well established.

We hypothesize that there is inter-individual variation in the action of some genes involved in TCDD effect in human cells.

Ethoxyresorufin-O-Deethylase (EROD) activity is measured as surrogate of the Aryl Hydrocarbon Hydroxylase (AHH) activity[2]. AHH, a product of the CYP1A1 locus, is involved in polycyclic aromatic hydrocarbon metabolism and, during this process, transforms procarcinogens to reactive carcinogenic intermediates[3]. CYP1A1 gene is up-regulated by certain foreign chemicals such as TCDD[4]. The induction of CYP1A1 gene requires the binding of the inducer to an intracellular protein, the Ah receptor, followed by the translocation of the complex to the nucleus and the binding of the liganded receptor to a specific DNA recognition sequence[5]. The mechanism by which the receptor-enhancer interaction activates gene transcription is not well understood. This process or the structural and functional properties of the Ah receptor could be different among individuals and determine different levels of CYP1A1 inducibility.

The quantity of Ah receptor, the CYP1A1 genotype and the EROD or AHH activities are susceptibility markers which may identify subjects who are at elevated risk for TCDD-related diseases.

The Seveso population, exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin in 1976, after an accidental explosion in a chemical plant 16 miles north of Milan,Italy[6], provides a unique opportunity for an epidemiologic investigation of this hypothesis. In fact, TCDD levels in exposed subjects are the highest recorded [7] and exposure is relatively free of other contaminants[8].

We designed a case-control study to examine a variety of susceptibility markers in 150 cancer cases and 150 controls in the Seveso population. We report here the results of a methodologic pilot phase designed to validate the assays planned for the case-control study. Intraindividual variation, inter-individual variation, reproducibility, stability under storage, shipping and the influence of potential confounders are the objectives of this phase. We have investigated a group of healthy men and women to determine if MspI polymorphism of

the CYP1A1 gene[9], studied in genomic DNA from lymphocytes of venous blood, correlates with different level of EROD activity and Ah receptor expression in those same lymphocytes. In particular we wanted to define whether the rare CYP1A1 genotype is associated with EROD activity and if there is a relationship with mRNA expression of the CYP1A1 gene (as a direct measurement of the gene inducibility[10]) and the Ah receptor. The CYP2D6 locus genotype was also studied in order to explore possible associations[11].

20 volunteers, aged 25-55 (roughly equal numbers of men and women) from the Environmental Epidemiology Branch of NCI were invited to participate. The subjects signed an informed consent, completed a short questionnaire about previous and recent smoking habits, grilled or broiled meat consumed in the last week, medication and vitamin use, and medical illness. 45 ml of blood was collected from each subject.

Care in collecting, storing, shipping and processing the blood samples was of utmost importance in this validation phase. EROD activity and Ah receptor expression measures require cells' isolation within a few hours of blood drawing. In a subgroup of samples lymphocytes cryopreservation was conducted with two different methods (Leuco-prep, Accuspin) to determine the most feasible for the Seveso study. The Accuspin method was selected.

The Ah receptor analysis was performed on 10 samples. Three of the samples yielded insufficient amount of receptor to do the assay. The receptor analysis was done on 200 μg of cytosolic protein and photoaffinity labelling with 2nM ^{125}I-2-azido- 3-iodo-7,8-dibromodibenzo-p-dioxin[12]. A 104 kDA band specific for affinity labeling was detected.

A PCR method was used to amplify a 340 bp fragment surrounding an MspI polymorphic locus 3' downstream to CYP1A1. Enzymatic digestion with MspI revealed the rare site present alleles when analysed with agarose gel (2.2%) electrophoresis.[13]. EROD activity and mRNA expression of the CYP1A1 gene using Northern Blot analysis with ^{32}P were measured both in basal and induced conditions. RNA quantity and purity were determined by A_{260}/A_{280} spectrophotometric absorbances. Integrity of RNA samples was confirmed by randomly selecting samples for gel electrophoresis in 1% agarose with visualization by ethidium bromide staining. The CYP2D6 genotype was characterized using a PCR test for the exon 5 deletion allele (A) and for the more common intron 3/exon 4 boundary mutation (B).

The statistical analysis was limited by the small number of subjects. Global indicators of smoking habit and meat consumption have been derived using a linear combination of the information collected by questionnaire.

Using multiple regression analysis (with the SAS package), RFLP of the CYP1A1 gene was statistically associated with basal and induced EROD activity (table 1). Meat consumption seemed to play some role (of borderline statistical significance) on induced EROD activity. Basal and induced mRNA expression were not associated with any of the variables considered.

Mean levels of EROD activity within the different CYP1A1 genotypes are presented in table 2. Basal EROD activity mean levels were statistically different in subjects with wt/wt and wt/mut CYP1A1 genotype. Induced EROD activity in wt/wt subjects was statistically different from subjects with wt/mut and mut/mut CYP1A1 genotypes. Putting together the genotypes with one or two mutations, both basal and induced EROD activity mean levels were statistically different from the wild type genotype. No difference was found in mean levels of mRNA expression among the different genotypes. The findings are consistent with a difference in the MspI determined CYP1A1 alleles, such that the mutants produce a protein which is quantitatively similar but qualitatively different[14].

This validation phase was successful in defining the methodologic procedures and in verifying the feasibility of a variety of laboratory assays, which have only been partially characterised in human populations. The preliminary results of this phase are also consistent with the hypothesized different level of EROD activity among subjects with different

CYP1A1 genotypes. The results will be used to refine questionnaire instruments and to focus on specific hypotheses for testing in the larger study.

TABLE 1

Multiple correlation (R^2) for basal and induced EROD activity (picomoles ethoxyresorufin/mg*min)

EROD activity	RFLP	RFLP+MEAT	RFLP+MEAT+OTHERS*
Basal	0.21	0.34	0.43
Induced	0.47	0.70	0.74

* Others includes: smoking habit, passive smoking exposure, and body mass index

TABLE 2

Mean levels and standard deviations of EROD activity (picomoles ethoxyresorufin/mg*min) within the CYP1A1 genotypes

CYP1A1 genotype	N	mean	stdv	T-test	p-value
Basal EROD activity					
wt/wt	11	0.30	0.09	3.005	0.0084
mutants	7	0.44	0.10		
wt/mut	5	0.46	0.11		
mut/mut	2	0.38	0.04		
Induced EROD activity					
wt/wt	11	2.49	0.77	5.675	0.0001
mutants	7	4.52	0.70		
wt/mut	5	4.70	0.53		
mut/mut	2	4.09	1.12		

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