UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

Dipartimento di Patologia e Sanita' Animale

DOTTORATO DI RICERCA IN

"BIOLOGIA , PATOLOGIA ED IGIENE AMBIENTALE IN MEDICINA VETERINARIA"

XXIII CICLO

COORDINATORE
PROF. GIUSEPPE PAINO

OSSERVAZIONI SULL'ATTIVAZIONE DEL RECETTORE ARILICO (AHR) IN CORRELAZIONE ALLA CONTAMINAZIONE AMBIENTALE DA METALLI PESANTI IN TUMORI SPONTANEI DI CANI DELLA REGIONE CAMPANIA.

"Correlation between Aryl hydrocarbon Receptor (AhR) expression and environmental heavy metals contamination in canine tumors."

Tutor :

Prof.ssa Serenella Papparella

Candidato:

Dott.ssa Francesca Trapani

A.A 2009/2010

Index

Introduction

In recent decades several studies have reported the effects of waste pollution on health, but in this precise period it seem very important to correlate the “waste problem” and its consequences in Campania , Region of Southern Italy, that has been a scene of controversy concerning waste disposal and treatment.

A wide range of toxic substance can be released into environment from waste disposal, for example, methane, carbon dioxide, benzene and heavy metals.

Many of these pollutants have been shown to be toxic for human health, in particular, two main health outcome have been found to be statistically associated with waste exposure: cancer and congenital malformation.

Hazardous waste has been show to influence the likelihood of developing bladder and lung cancer,

and living close a waste disposal site is also associated with a significant increase in congenital anomalies.(1) (2)

During 2010, ARPA (Regional Agency for Environmental Protection) has identified and characterized the various authorize/unauthorized dumping sites in the provinces of Naples and Caserta, in order to locate the possible areas exposed to a higher waste related health risk.

As a first result , some municipalities along the coast and north of Naples have been shown to be characterized by high-risk impact areas.(3)(Fig.1)

It's important to underline that Naples's harbor is one of the largest and most important commercial and touristic port of Mediterranean basin and receives industrial and municipal wastewater from the city of Naples.

For this reason, many other factor , as extensive agriculture, industrial activities, high population density, influence the territory from an environmental point of view and have to be examined in the context of a multivariate analysis.

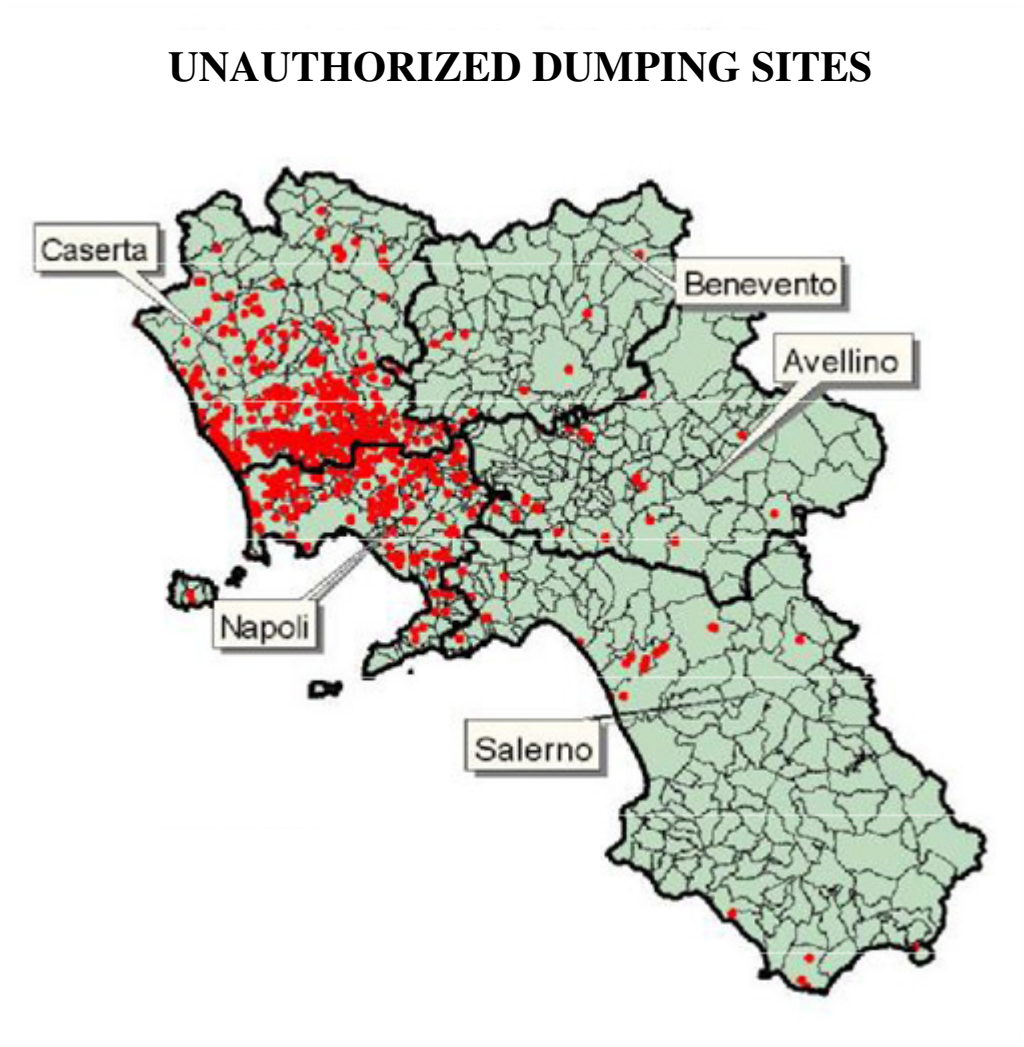


Fig.1 The unauthorized dumping sites in Campania Region.

1.1 **Bioavailability and hazard of heavy metals contamination.**

Emissions of heavy metals to the environment occur through a wide range of processes and pathways, including to the air (e. g during combustion) or to surface waters and to the soil, but all type of municipal solid waste contain more heavy metals than the background concentration present in soil.

Even if heavy metals have been used in many different areas for thousands of years, in the last two decades has emerged a new source of contamination: the electronic waste.(E-waste).

E-waste is a critical global environmental health issue because of its massive production volume and insufficient management policy in many countries.

E-waste contains heavy metals and persistent organic pollutants (POPs) and inappropriate recycling processes occurring in several developing countries result in the release of these toxicants into the environment (4).

E-waste is the fastest growing stream of municipal solid waste but its management is a significant environmental health concern.

It is estimated that 20-50 million tons of e-waste are produced annually worldwide; the United States, Western Europe, China, Japan, and Australia are the major producers (UNEP 2005).

According to a U.S. Environmental Protection Agency (US EPA) estimate, the U.S. generated

approximately 2.5 million tons of e-waste in 2007, which accounts for about 2% of municipal

solid waste and has a projected annual increase of 3-5% (US EPA 2008).

In the U.S., only about 20% of E-waste is collected for recycling, with the remaining 80% sent to landfill. (US EPA 2007, 2008).

Actually these dates are not evaluable in Italy.

Landfill can cause metal leaching from the E-waste and burning e-waste may produce extremely toxic dioxins and furans (6)

Developing countries are generating more and more E-waste in their own territories and may also feed the recycling business (4).

However, because of a lack of stringent environmental regulation and worker protection, toxicants in E-waste cause serious contaminations of local air, dust, soil, and water (7).

The environmental consequence is dire in these regions if the activities remain uncontrolled. Further, informal recycling processes (dismantling, cutting, heating, acid leaching, and burning) expose the workers and residents to dangerous mixtures of metals and other pollutants (4)(8).

Several biological mechanisms (oxidative stress, neuroendocrine disruption and epigenetic modifications) are involved in cancer development but data are insufficient to address the problem to exposure mixtures such as in E-waste, and more research is needed to investigate the combinations of effects of heavy metals and cancer

Epigenetic modifications are mitotically heritable changes of gene function in the absence of alterations in nucleotide sequence.

These epigenetic changes include DNA methylation, mostly in the 5'-cytosine in the CpG dinucleotides of the gene promoter region, modifications, and micro RNAs (miRNAs) that affect posttranscriptional regulation (9).

Because nucleotide sequence is generally static in somatic cells and epigenetic markers are modifiable during the life course, the investigation of epigenetic changes induced by environmental toxicants has received increased attention (9).

The aim of this work is to investigate the correlation between environmental contamination by heavy metals and cancer development in dogs lived in Campania region.

To assess the presence of this correlation we speculate the expression of Aryl hydrocarbon receptor (AhR) and its enzymatic pathway (P450 Pathway) in canine tumors by immunohistochemistry methods and ICP- MS technology .

1.2 Aryl hydrocarbon receptor : mechanism and action.

The Aryl hydrocarbon receptor (AhR) was discovered as a results of a program to understand the basis of chemical carcinogenesis.

At the beginning , while investigating the ability of the polycyclic aromatic hydrocarbon (PAH) to cause ulceration in mouse skin and induce cytochrome P450 1A1 (CYP1A1), it was found that a single gene locus controlled the responsiveness of different mouse strains to polycyclic compounds (10)(11).

This was designated the Ah (short for aryl hydrocarbon) locus.

The Ah locus controlled the induction of cytochrome P450 to metabolized PAHs to genotoxic metabolites, thereby showing a key role for Ah in skin ulceration and carcinogenesis.

It's generally accepted that the toxic responses of environment pollutants are the direct consequences of AhR activation.

The AhR is a transcriptional factor belonging to the helix-loop-helix/Per/ARNT/Sim (PAS) family.

The human AhR encodes a protein of 848 amino acids , of which 10 are cleaved from N-terminus.

Notable domains include an N-terminal basic helix-loop-helix domain, involved in binding to DNA, a C-terminal transcription activation domain, and two "PAS" repeats in the middle of the protein which include the region of the molecule that binds to the ligands.

PAS protein are a superfamily of conditional transcription factor containing a PAS sequence; the term is derived from the founding members of this superfamily namely **Per** (Period regulator of circadian rhythms) **Arnt** (Aryl hydrocarbon receptor nuclear translocator) and **Sim** (single minded regulator of midline cell differentiation).

The Ah protein require a complex folding pathway involving the chaperones, heat shock protein 90 (HSP90) and AhR- interacting protein (AIP) among other to achieve the functional ligand –binding form of the receptor.

Removing of HSP90 or AIP from cells results dysfunctional AhR, so it is known that these are required for correctly folding the AhR.

This complex folding pathway results in low levels of expression of ligand-binding competent AhR, which is a major difficulty in establishing the structural basis of ligand - binding to the Ahr. (12)

The correctly folded AhR is located in cytosol of cells with chaperone, until it binding to a ligand.

(FIG.2)

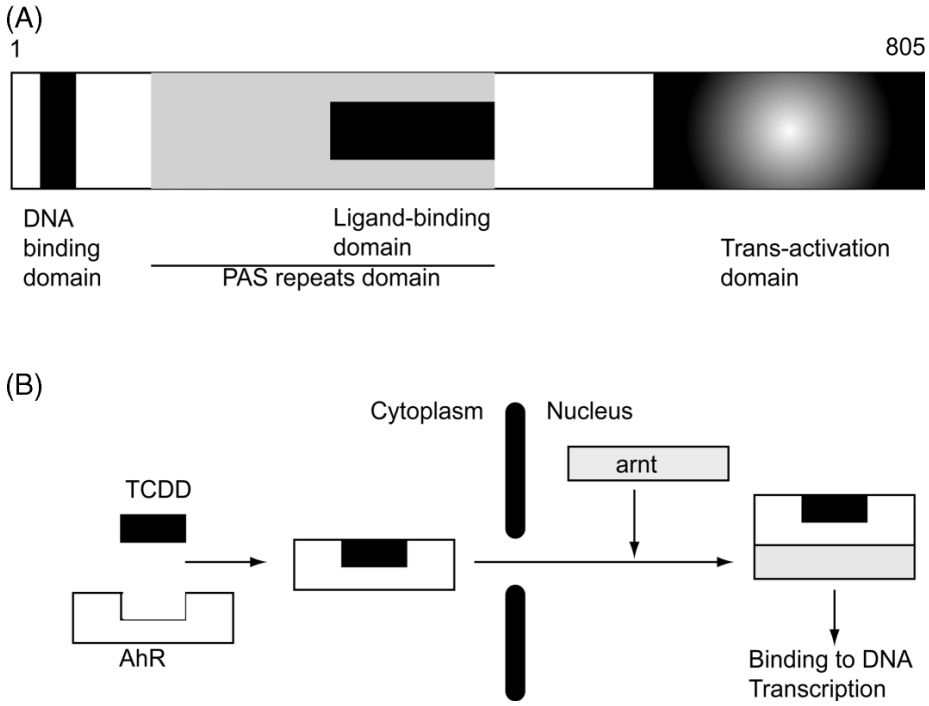


Fig. 2. (A) Cartoon of the primary structure of the Ah receptor. The mouse AhRb−1 receptor is shown as an 805 amino acid structure. The basic helix–loop–helix DNA-binding structure is shown as a black bar, and the two Per–Arnt–Sim (PAS) domain repeats are shown as a light gray box, enclosing a black box identifying the minimal residues necessary for ligand binding.

The C-terminal transactivation domain is shown as a black- and gray-shaded region.

(B) Schematic of AhR action. TCDD diffuses into the cell and binds to cytoplasmic AhR.

The AhR then translocates into the nucleus, whereupon it binds to Arnt.

The AhR–arnt dimer then causes transcription of target genes.

Tha AhR translocated to the nucleus , dissociating from the chaperons, and forme a dimeric complex with another PAS family protein ,**arnt.**

The **Ahr – arnt** complex then binds to particular sequences in DNA , known as xenobiotic response elements (XRE), and cause s transcription of specific genes

encoding phase I and II xenobiotic metabolizing enzyme such as cytochrome P450s (CYP1A1, CYP1A2, CYP1B1).

Studies in knockout mice indicate that CYP1A1 is predomintly important for detoxification.(13)

The ligand-bound, active AhR is tightly regulated by the cell and is subject to rapid proteolytic degradation, so >90% of nuclear AhR is exported from the nucleus and degradated within 4h of treatment with ligand (14)

Since carcinogenicity of PAHs is lost in AhR knockout mice, it's generally accepted that this canonical AhR - dipendent pathway is required for tumor initiation by PAHs.

Mice expressing a constitutively active AhR show an increase in the development of stomach tumors implies that the activation of AhR leads to deregulation of cell cycle control in vivo.

The role of the tumor promoting effects of the AhR is still unclear.

Beside its functions in tumor initiation and tumor promotion, recent studies suggest that AhR also plays a role in tumor progression, for example in transition from benign to a malignant tumor.

For instance, AhR expression is higher in invasive than in non – invasive tumour cell and tissue.

The upregulation of nuclear AhR in human urothelial tumor is associated with increase invasion and poor prognosis.

Importantly, fibroblast derived from AhR knockout mice show decreased tumorigenicity and migration due to down-regulation of the proto-oncogene Vav3.

By contrast, activation of AhR leads to cell cycle arrest in G1 phase in several cell lines.

Cell-cell contact is known to be a critical regulator of cellular proliferation and motility.

Inhibition of proliferation by cell-cell contact is generally referred to as contact inhibition or contact dependent inhibition of growth.

Vice versa, release from contact inhibition is associated with abnormal cellular proliferation and tumor promotion is characterized by unbalanced proliferation due to increase proliferation or decreased level of apoptosis.

Moreover, loss of proper cell-cell adhesion is the sine qua non condition for tumor progression.

The activation of AhR leads to deregulation of contact inhibition.

Recent studies have shown AhR signaling pathway up regulates the expression of matrix metalloproteinase (MMP): the AhR pathway also mediated the plasminogen activator inhibitor-2 and urokinase type plasminogen.

The plasmin system can activate several MMPs by cleaving the prodomain.

It was demonstrated that AhR activated by TCDD contributed to melanoma progression, specifically through the stimulation of the progression of MMP-1, MMP-2 and MMP9. (15)

1.3 **Classification of P450s**

The cytochromes P450 (P450) are a multi-gene family of constitutive and inducible oxidative enzymes with an important role in the metabolism of a diverse range of xenobiotics. (16)

This group of enzymes is one of the largest known mammalian gene families and takes its name from the characteristic absorption peak of the protein at 450 nm when it is reduced and complexed with carbon monoxide.

The primary role that has been identified for P450s is the deactivation of a wide variety of environmental chemicals.

Indeed, the P450 system is considered to be as important in protecting the body from small molecular weight foreign compounds .

There is also increasing evidence that the P450s have endogenous functions (17).

Functionally, P450 is the terminal oxidase of the microsomal enzyme oxidizing system.

The P450s catalyse the stereospecific incorporation of oxygen into a wide range of substrates at physiological temperatures, using reduced nicotinamide dinucleotide phosphate (NADPH) as a cofactor.

These enzymes are very powerful catalysts, since in the absence of P450 this oxidation reaction requires a very high temperature to proceed and then only non specifically.

Usually the outcome of P450 metabolism is inactivation of the substrate; paradoxically, in some cases the consequence of P450 metabolism is activation to a reactive intermediate and this can lead to either acute or chronic cellular toxicity (18)
Structurally, the P450s consist of several domains. There is a hydrophobic N-terminus, which acts as a membrane anchor for the P450; a substrate-binding site; an oxygen binding site; and a free hydrophilic C-terminal.

The P450s are considered to have a central role in tumor development and progression and are involved in tumor initiation and promotion, since they can activate or deactivate most carcinogens (18)(19)

Furthermore, the P450s can influence the response of established tumors to anti-cancer drugs.

The P450s are classified into families, sub-families, and individual forms based on nucleic acid and amino acid sequence homology .

There are two broad groups of mammalian P450s, a large group whose primary role is the metabolism of xenobiotics (CYP1, CYP2, CYP3, and to a lesser extent CYP4), and a much smaller group of P450s which are constitutively expressed in endocrine glands, where they are specifically involved in steroid hormone synthesis (CYP11, CYP17, CYP19, and CYP21).

Those P450s that have been primarily characterized according to their ability to metabolize xenobiotics are also capable of metabolizing endogenous compounds.

This indicates that they may also have endogenous functions, particularly involvement in cell regulation and cell signaling. (20)

Among the CYP450s, CYP1A1 has received considerable attention because it is highly induced by a broad range of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) through the AhR–XRE transcription pathway (21)

CYP1A1 is capable of producing polar toxic or even carcinogenic metabolites from various AhR ligands, including PAHs and heavy metals

The regulation of CYP1A1 has been extensively studied, yet it is not completely understood.

Changes in physiological conditions, including stressful conditions such as hyperoxia and suspension of cells, or induction of differentiation, increase CYP1A1 expression in the absence of an exogenous ligand.

With the identification of an active AhR in cell culture and tissue slices , in the absence of an exogenous AhR ligand, it was proposed that a ligand-independent mechanism might be responsible for AhR activation and subsequent CYP1A1 induction (21).

The exact mechanisms governing the ligand-independent activation of AhR are still not clear.

However, it has been reported that activation of the cAMP mediator or MAPK signaling pathways increase AhR nuclear translocation.

Theories regarding ligand-independent activation of the AhR have been shadowed with the identification of a large number of endogenous compounds with the ability to activate the AhR in vitro.

These ligands have been grouped into several categories, including indoles, tetrapyroles and arachidonic acid metabolites (21).

Indole- containing substances are primarily endogenous metabolites of tryptophan.

Tryptophan and several of its naturally occurring metabolites, including tryptamine, indole acetic acid, indigo and indirubin, have been reported to activate the AhR in yeast and mammalian cell cultures (23).

Products of the heme degradation pathway have also been found to activate the AhR signaling pathway.

Bilirubin and biliverdin activate the AhR in cultured cells at physiologically relevant concentrations.

Hydrophobic products of arachidonic acid metabolism, most notably lipoxin A4 and prostaglandins (PGs), also activate the AhR .

In fact, several PGs, including PGG2, PGD3 and PGH1, induced DNA binding of the AhR complex in vitro.

Interestingly, these endogenous ligands seem to be relatively weak inducers compared to the classic inducers (i.e., PAHs and HAHs).(24)

The induction of CYP1A1 by this structurally distinct class of compounds brings into light several identifiable factors that can influence the capacity of an AhR ligand to induce CYP1A1.

AhR affinity plays a major role in determining the expression level of CYP1A1.

This is illustrated in the C57BL/6 mice, which are sensitive to PAHs and HAHs and the DBA/2 mice, which possess a lower affinity AhR and thus have lower induced CYP1A1 levels (> 15-fold difference)(25).

Heavy Metals

Heavy Metals, a major category of globally-distributed pollutants, are natural elements extracted from the earth and harnessed for human industry and products for millennia.

Metals are notable for their wide environmental dispersion from such activity; they tend to accumulate in select tissues of the human body and can be toxic even at relatively minor levels of exposure.

Some metals, such as copper and iron, are essential to life and play irreplaceable roles, for example, the functioning of critical enzyme systems.

Other metals are xenobiotics, i.e., they have no useful role in human physiology (and most other living organisms) and, even worse, as in the case of lead and mercury, may be toxic even at trace levels of exposure.

Even those metals that are essential, however, have the potential to turn harmful at very high levels of exposure, a reflection of a very basic tenet of toxicology--"the dose makes the poison."

One reflection of the importance of metals relative to other potential hazards is their ranking by the U.S. Agency for Toxic Substances and Disease Registry (ATSDR), which lists all hazards present in toxic waste sites according to their prevalence and the severity of their toxicity.

Among all heavy metals, As^{3+}, Hg^{2+},Cr^{3+}, Pb^{2+} and Cd^{2+} are ranked the highest.

Exposure to metals can occur through a variety of routes.

Heavy metals may be inhaled as dust or fume (tiny particulate matter, such as the lead oxide particles produced by the combustion of leaded gasoline).

Some metals can be vaporized (e.g., mercury vapor in the manufacture of fluorescent lamps) and inhaled.

Metals may also be ingested involuntarily through food and drink, the amount absorbed from the digestive tract can vary widely, depending on the chemical form of the metal and the age and nutritional status of the individual.

Once a metal is absorbed, it distributes in tissues and organs.

Excretion typically occurs primarily through the kidneys and digestive tract, but metals tend to persist in some storage sites, like the liver, bones, and kidneys for years or decades.

Some metals, such as arsenic, are clearly capable of causing cancer.

2.1 Lead

For centuries, lead has been mined and used in industry and in household products.

The dominant source of worldwide dispersion of lead into the environment (and into people) for the past 50 years has clearly been the use of lead organic compounds as antiknock motor vehicle fuel additives.

Since leaded gasoline was introduced in 1923, its combustion and resulting contamination of the atmosphere has increased background levels everywhere.

The current annual worldwide production of lead is approximately 5.4 million tons and continues to rise.

Sixty percent of lead is used for the manufacturing of batteries (car batteries, in particular), while the remainder is used in the production of pigments, glazes, solder, plastics, cable sheathing, ammunition, gasoline additive, and a variety of other products.

Such industries continue to pose a significant risk to workers, as well as surrounding communities.

In response to these risks, many developed countries over the last 25 years have implemented regulatory action that has effectively decreased actual exposures to the general population.

However, exposures remain high or are increasing in many developing countries through a rapid increase in vehicles combusting leaded gasoline and polluting industries.

Moreover, some segments of the population in developed countries remain at high risk of exposure because of the persistence of lead paint, lead plumbing, and lead-contaminated soil and dust, particularly in areas of old urban housing.

A number of factors can modify the impact of lead exposures, for example, water with a lower pH (such as drinking water stemming from the collection of untreated "acid rain") will leach more lead out of plumbing connected by lead solder than more alkaline water.

Lead from soil tends to concentrate in root vegetables (e.g., onion) and leafy green vegetables (e.g., spinach).

Individuals will absorb more lead in their food if their diets are deficient in calcium, iron, or zinc.

Other more unusual sources of lead exposure also continue to be sporadically found, such as improperly glazed ceramics, lead crystal, imported candies, certain herbal folk remedies, and vinyl plastic toys.

Experimental evidence suggests that inorganic lead is weakly mutagenic but only at high toxic doses .

However, it has indirect genotoxic effects when administered in combination with other DNA-damaging agents, suggesting a possible co-carcinogenic effect.

This probably occurs when lead ions interact with enzymes implicated in DNA processing and repair.

Experimental studies suggest increased incidences of kidney cancer, and to a lesser extent lung, brain and hematopoietic cancers, among rodents exposed to inorganic lead .

Less is known about the carcinogenicity of organic lead.

Organic lead is absorbed through the respiratory tract and skin more efficiently than is inorganic lead. It is partly metabolized into ionic lead, which is expected to harbor the carcinogenic properties of inorganic lead .

Several studies have focused on cancer risk among workers exposed to lead in industries such as battery manufacturing, smelting, and pigment production, where exposures to mainly inorganic lead compounds occur at high levels.

Results from such studies and meta-analyses have suggested that there may be excess risks for lung and stomach cancers among these workers. Most studies suggestive of a link between lead exposure and stomach cancer have focused on inorganic lead.(Occupational Exposure to Lead Compounds and Risk of Cancer among Men: A Population-based Case-Control Study)

2.2 Mercury

Mercury comes in a number of different chemical forms.

Metallic mercury is used in thermometers, dental amalgams, and some batteries.

In its pure form, metallic mercury is a liquid.

Mercurous and mercuric mercury (Hg^+ and Hg^{2+} , respectively) are encountered in some chemical, metal-processing, electrical-equipment, automotive, and building industries and in medical and dental services.

Mercurous and mercuric mercury form inorganic and organic compounds with other chemicals that can be readily absorbed through ingestion.

All three forms of mercury are toxic to various degrees.

From a global perspective, mercury has been increasing in importance as a widespread contaminant. About half of the National Priority List toxic waste sites in the U.S. contain mercury.

Mercury dispersion through atmospheric deposition has increased markedly through waste incineration; ironically, the medical industry is one of the largest contributors to mercury pollution.

Some countries, such as Brazil, have seen widespread mercury contamination (and resultant health effects) through a combination of its indiscriminate use in gold mining and deforestation. When deposited in soil, organic mercury compounds are slowly broken down into inorganic compounds; conversely, inorganic mercury can be converted by microorganisms in soil and water into the organic compound methyl mercury, which is then bioconcentrated up the food chain.

Fish, particularly tuna, king mackerel, and swordfish, can concentrate methyl mercury at high levels.

No human data indicate that exposure to any form of mercury causes cancer, but the human data currently available are very limited. Mercuric chloride has caused increases in several types of tumors in rats and mice, and methylmercury has caused kidney tumors in male mice. Scientists only observed these health effects at extremely high doses, above levels that produced other effects.

2.3 Arsenic

Significant exposure to arsenic occurs through both anthropogenic and natural sources.

Occupational and community exposures to arsenic from the activities of humans occur through the smelting industry, the use of gallium arsenide in the

microelectronics industry and the use of arsenic in common products such as wood preservatives, pesticides, herbicides, fungicides, and paints.

In some areas of the world, arsenic is also a natural contaminant of wells.

Deep-water wells in parts of Taiwan and Chile are now well-known to be contaminated with arsenic, giving rise to chronic manifestations of toxicity.

Water from relatively shallow tube wells that were placed in areas of Bangladesh, West Bengal, and other parts of the subcontinent has also been recently found to be heavily contaminated with arsenic.

Water in some parts of the U.S., such as areas of the Southwest, also carry a significant risk of arsenic contamination.

The toxicity of an arsenic-containing compound depends on its valence state, its form (inorganic or organic), and factors that modify its absorption and elimination.

Inorganic arsenic is generally more toxic than arsenic, and trivalent arsenite is more toxic than pentavalent and zero-valent arsenic.

These nuances are important. For example, testing biological samples for arsenic in an individual with suspected toxicity must be done more than 48 hours after the individual abstains from eating seafood; otherwise, the test may be confounded by the presence of arsenobentaine, a relatively harmless form of arsenic that is contained in fish at high levels of concentration.

Once absorbed into the body, arsenic undergoes some accumulation in soft tissue organs such as the liver, spleen, kidneys, and lungs, but the major long-term storage site for arsenic is keratin-rich tissues.

Acute arsenic poisoning is famous for its lethality, which stems from arsenic's destruction of the integrity of blood vessels and gastrointestinal tissue and its effect on the heart and brain.

Chronic exposure to lower levels of arsenic results in somewhat unusual patterns of skin hyperpigmentation, peripheral nerve damage manifesting as numbness, tingling, and weakness in the hands and feet, diabetes, and blood vessel damage resulting in a gangrenous condition affecting the extremities.

Chronic arsenic exposure also causes a markedly elevated risk for developing a number of cancers, most notably skin cancer, cancers of the liver (angiosarcoma), lung, bladder, and possibly the kidney and colon.

Studies to date have shown that individuals who live in arsenic contaminated areas of the world exhibit an elevated cancer rate.

While the correlation between exposure to arsenic resulting in human tumors such as those derived from bladder, lung and skin is well established, the molecular mechanisms driving this connection is unclear.

Using experimental data from cell cultures and results of epidemiologic studies, the researchers found that arsenic activates several pathway that indirectly leads to cancer.

2.4 Cadmium

Cadmium exposure is encountered in industries dealing with pigment, metal plating, some plastics, and batteries.

Cadmium pollution (e.g., the emissions of a cadmium smelter or industry and the introduction of cadmium into sewage sludge, fertilizers, and groundwater) can result in significant human exposure to cadmium through the ingestion of contaminated foodstuffs, especially grains, cereals, and leafy vegetables.

Airborne cadmium exposure is also a risk posed by the incineration of municipal waste containing plastics and nickel-cadmium batteries. Cigarette smoking constitutes an additional major source of cadmium exposure.

The health implications of cadmium exposure are exacerbated by the relative inability of human beings to excrete cadmium. (It is excreted but then re-absorbed by the kidney.)

Acute high-dose exposures can cause severe respiratory irritation.

Occupational levels of cadmium exposure are a risk factor for chronic lung disease (through airborne exposure) and testicular degeneration and are still under investigation as a risk factor for prostate cancer.

Lower levels of exposure are mainly of concern with respect to toxicity to the kidney. Cadmium damages a specific structure of the functional unit of the kidney (the proximal tubules of each nephron) in a way that is first manifested by leakage of low molecular weight proteins and essential ions, such as calcium, into urine, with progression over time to frank kidney failure.

Even without causing frank kidney failure, however, cadmium's effect on the kidney can have metabolic effects with pathologic consequences. In particular, the loss of calcium caused by cadmium's effect on the kidney can be severe enough to lead to weakening of the bones.

"Itai itai" disease, an epidemic of bone fractures in Japan from gross cadmium contamination of rice stocks, has recently been shown to happen in more subtle fashion among a general community living in an area of relatively modest cadmium contamination.

Cadmium exposure has been associated with human prostatic cancer in some, but not all, epidemiologic studies.

Some studies indicate that tissue levels of cadmium in the human prostate correlate with malignant disease.

Any association between cadmium and prostatic cancer has been controversial, in large part because of a previous lack of relevant animal models.

However, several chronic studies in rats revealing a correlation between cadmium exposure and prostatic tumors have been published over the last several years.

Moreover cadmium increases breast cancer cell proliferation in vitro by enhancing Akt, ERK1/2 and PDGFRα kinases activity.

2.5 Chromium

Occupational exposure to Cr (VI) compounds in a number of industries has been associated with increased risk of respiratory system cancers.

The first epidemiological study of chromate production workers in the United States that demonstrated an association with lung cancer was conducted with 1,445 workers in seven plants engaged in the extraction of chromates from ore from 1930 to 1947.

The percentage death due to cancer of the respiratory system was 21.8%; the percentage expected was 1.4%.

In addition to lung cancer, a number of epidemiological studies of workers in chromate industries also showed significantly increased risk for nasal and sinus cancers .

On the basis of these and other studies, the U.S. Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC) have classified inhaled Cr(VI) as a known human carcinogen [IARC 1990; EPA 1998].

The World Health Organization (WHO) has determined that Cr(VI) is a human carcinogen. The Department of Health and Human Services (DHHS) has determined that Cr(VI) compounds are known to cause cancer in humans [ATSDR 2000].

Lung cancer risk in relation to airborne levels of Cr (VI) was analyzed for chromium chemical production workers and a dose-response relationship was observed in that long-term workers had a higher lung cancer risk than short-term workers.

An analysis of lung cancer risk suggests a potential excess risk of death from lung cancer among U.S. workers exposed to the previous permissible exposure limit (PEL) for Cr(VI) of 52 µg/m³ .

Stratified analysis of lung cancer mortality showed a trend of increasing mortality with higher cumulative exposure levels. The analyses stratified by duration of employment and time since first exposure indicate a consistency of results among those employed the longest and with the longest elapsed time since first exposure. The latter suggests a latency period of approximately 20-35 years, which is compatible with other research.

Carcinogenicity appears to be associated with the inhalation of the less soluble/insoluble Cr(VI) compounds. The toxicology of Cr(VI) does not reside with the elemental form.

Epidemiological evidence strongly points to Cr(VI) as the agent in carcinogenesis.

Solubility and other characteristics of chromium, such as size, crystal modification, surface charge, and the ability to be phagocytized, compounds might be important in determining cancer risk.

A number of chronic inhalation studies provide evidence that Cr(VI) is carcinogenic in animals [ATSDR 2000].

The mechanisms of Cr(VI)-induced carcinogenicity is not completely understood.

The toxicity of chromium within the cell may result from damage to cellular components during the hexavalent to trivalent chromium reduction process, by generation of free radicals, including DNA damage [ATSDR 2000]. Recent studies indicate a biological relevance of non-oxidative mechanisms in Cr(VI) carcinogenesis.

AhR- Cytochrome P450 and Heavy metals: regulatory mechanism.

Korashy et al. (2005) provide the evidence that heavy metals modulate CYP1A1 gene expression at transcriptional and post-transcriptional levels.

All metals induce CYP1A1 mRNA in a time dependent manner and cause the activation of the AhR.

To understand the relation between CYP1A1 and heavy metals induction a series of experiments were carried out.

The transcriptional regulation of CYP1A1 gene expression by heavy metals was demonstrated through different approaches: first the inhibition of RNA transcription, using actinomycin D, completely abolish the induction of CYP1A1 mRNA in response to heavy metals.

Second co-admistration of metals with cyclohexamide (an inhibitor of protein biosynthesis) superinduced the CYP1A1 mRNA in response to metals.(31)

According with these experiments was postulated that heavy metals may bind to the

AhR-associated protein , resulting in AhR conformational changes which lead to activation of the receptor and its translocation to the nucleus. (31)

In have recently shown that heavy metal-induced oxidative stress plays a role in the transcriptional induction of the CYP1A1 gene and the post translational inhibition of CYP1A1 activity .

The non-persistent effect of the metals is likely due to their cellular clearance by metabolic and/or oxidative mechanisms (27).

The cellular mRNA level, at any point in time, is a function of the rate of its production, by transcriptional mechanisms, and the rate of its degradation.

It is apparent that As3+, Cd2+ and Cr6+ induce mRNA expression by increasing the rate of transcription of the CYP1A1 gene and decreasing the degradation of the mRNA transcripts.

The ability of the metals to activate AhR-dependent gene expression may suggest an interaction of the metals with the AhR.

The majority of these compounds however are weak inducers, which explains the relatively weak induction of CYP1A1 mRNA.

Metals may stabilize the CYP1A1 protein by direct binding to the apoprotein, rendering it less susceptible to proteases.

Alternatively, the reduction in CYP1A1 activity may be a consequence of cross talk among stress-responsive signaling pathways that are activated as part of a cell's response to limit intracellular damage (29).

In particular, numerous studies have shown that Cd2+ exposure affects total hepatic CYP450 and monoxygenase activities in different mammalian systems.

The most interesting findings with regard to the effect of Cd2+ on CYP1A1 are that Cd2+ alone was able to induce CYP1A1 mRNA and was also shown to be a potent inducer of AhR nuclear accumulation.

Cd2+ did not affect Cyp1a1 mRNA stability, indicating a predominant transcriptional mechanism for the increase in Cyp1a1 mRNA.

Although the effect of As3+ on CYP1A1 activity does not always parallel its effect on the expression on CYP1A1 mRNA, almost all studies have reported a decrease in CYP1A1 activity in hepatic tissue or cells.

It seems that As3+ may have a direct effect on the function of the CYP450 protein, independent of transcriptional regulation.

As such, it has been well documented that As3+ interacts with critical cysteine residues of many intercellular proteins, thus, altering their functions.

As3+ has been shown to stimulate the production of superoxide (O2-) and hydrogen peroxide as a result of its intrinsic ability to accumulate in the mitochondria and alter cellular respiration

The mechanisms by which As3+ induces CYP1A1 mRNA transcription through the AhR remain unresolved. Heavy metals may alter some cellular metabolic pathways leading to the enhanced production of endogenous AhR ligands.

Experimental Investigation

To evaluated the association between the expression of AhR and CYP1A1, and correlation with heavy metals levels in tumors, we performed an immunohistochemical analysis of 32 samples

extract from cases database from the Department of Pathology and Animal Health, Veterinary Faculty of Federico II University, Naples (Italy).

The cases were chosen according to their geographical provenience, 22/32 cases coming from an area in Naples with high volume of traffic, dumps and waste deposition and 10/32 cases coming from Salerno's area with low pollution risk. (Tab.1)

Methods:

All the tissue were fixed in 10% formalin and embedded in paraffin.

After morphological evaluation on haematoxylin/eosin staining slides, sections, 4 µm thick, were used for immunohistochemistry.

After deparaffination in xilene and dehydratation in a graded ethanol series, an antigen retrivial procedures was performed by heating the slides in 10 mM citrate buffer (pH 6.0).

The endogenous peroxidise activity was blocked by incubation with 0.3% hydrogen peroxidise/methanol for 15 min.

After incubation with protein block serum free (Dako), the section were incubate at 4° overnight with a rabbit polyclonal anti-AhR antibody (1:100 dilution, Sc-5579, Santa Cruz Biotechnology, CA) and with a rabbit polyclonal anti CYP1A1 (H70) (1.100 dilution, Sc-20772, Santa Cruz Biotechnology, CA).

After washing with phosphate buffered saline they were developed by Strepdavin /Biotin system (Dako) with Diaminobenzamina tetrahydrochloride (DAB).

The section were counterstained with haematoxylin.

Contaminant analysis

Trace elements.

Before analysis, whole bodies were lyophilised and homogenised; aliquots of 100 mg were weighed and digested in microwave oven with HNO3 and H2O2 ultrapure in PFA vessels.

Digested samples were transferred to polyethylene tubes and diluted to20 ml with ultra-pure water.

Metal concentrations in samples were determined by ICP-MS (inductively coupled plasma mass spectrometry) with collision cell (He) to remove the interferences. The method of external calibration was used and standards were prepared by dilution of commercial stock solutions to within the linear range of the respective metals.

Table 1

Diagnosi	Breed	Age/Years	Sex	Geographical area
1-Rectal Cancer	Crossbreed	10	M	Naples
2-Renal Carcinoma	German Sheperd	6	F	Cava dei Tirreni (Sa)
3-Rectal Cancer	Pechinese	14	F	Salerno
4-Urothelial Carcer	German Sheperd	11	F	Naples
5-Urothelial Carcer	Crossbreed	12	F	Naples
6-Urothelial Carcer	Crossbreed	11	F	Salerno
7-Renal Carcinoma	Crossbreed	11	F	Naples
8-Lung Cancer	Yorkshire	12	M	Salerno
9-Prostate cancer	German Sheperd	9	M	Pompei (Na)
10-Renal Carcinoma	German Sheperd	5	F	Battipaglia (Sa)
11-Anal Sac Adenocarc	German Sheperd	10	M	Salerno
12-Hepatic Cancer	Crossbreed	8	M	Naples
13-Hepatic Cancer	Crossbreed	11	F	Naples
14-Hemangiopericytoma	S.Husky	8	M	Naples
15-Solid Carcinoma	Crossbreed	8	F	Naples
16-Malignant Trichoepithelioma	Boucheron Shep.	6	M	Caivano(Na)
17-Keratoachantoma	Yorkshire	7	F	Caivano(Na)
18-Hemangiopericytoma	Crossbreed	10	F	Salerno
19-Mastocytoma	Crossbreed	10	M	Caivano (Na)
20-Gigant Cell osteosarcoma	S.Husky	9	F	Caivano (Na)
21-Mammary adenocarcinoma	S.Husky	5	F	Caivano (Na)
22-Mammary Carcinoma	Crossbreed	10	F	Caivano (Na)
23-Mammary adenocarcinoma	Crossbreed	5	F	Caivano (Na)
24-Anaplastic sarcoma	Dalmatian Sheperd	11	F	Naples
25-Fibrosarcoma	Dalmatian Sheperd	11	F	Naples
26-Hemangiosarcoma	German Sheperd	8	M	Naples
27-Anaplastic Sarcoma	Crossbreed	8	M	Caivano(Na)
28-Mixosarcoma	Pechinese	2	M	Naples
29-Neurofibrosarcoma	Crossbreed	10	M	Nocera Inf. (Sa)
30-Anal Sac Adenocarc	S.Husky	13	M	Amalfi (Sa)
31-Anal Sac Gland Adenoma	Crossbreed	4	M	Tramonti (Sa)
32-Normal Lung/Liver/Kudney	Crossbreed	1 month	M	Naples

.

Results

Because AhR acts as a transcriptional regulator with nuclear translocation we evaluated both the nuclear and the cytoplasmic AhR and CYP1A1 staining.

Cytoplasmic AhR and CYP1A1 staining were observed in the non-neoplastic tissue with a weak intensity also the nuclear AhR staining was also very weak or negligible

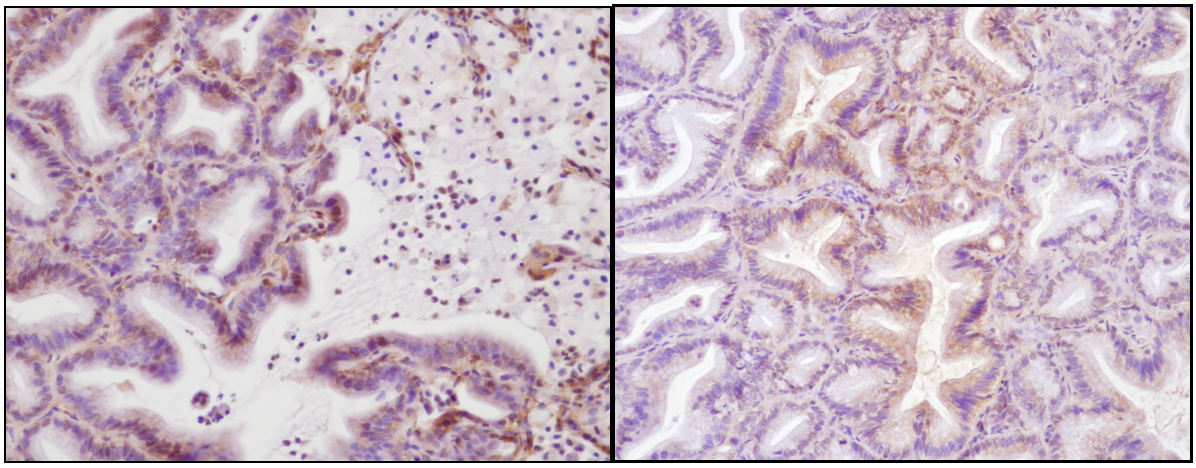
In the benign tumour samples, there was a moderate degree of cytoplasmic staining and weak nuclear AhR staining was observed in only a small number of the cancer cells.

In the invasive and malignant tumor sample , strong AhR/CYP1A1 staining were observed in many of the nuclei of the cancer cells, but the cytoplasmic AhR staining was rather weak.

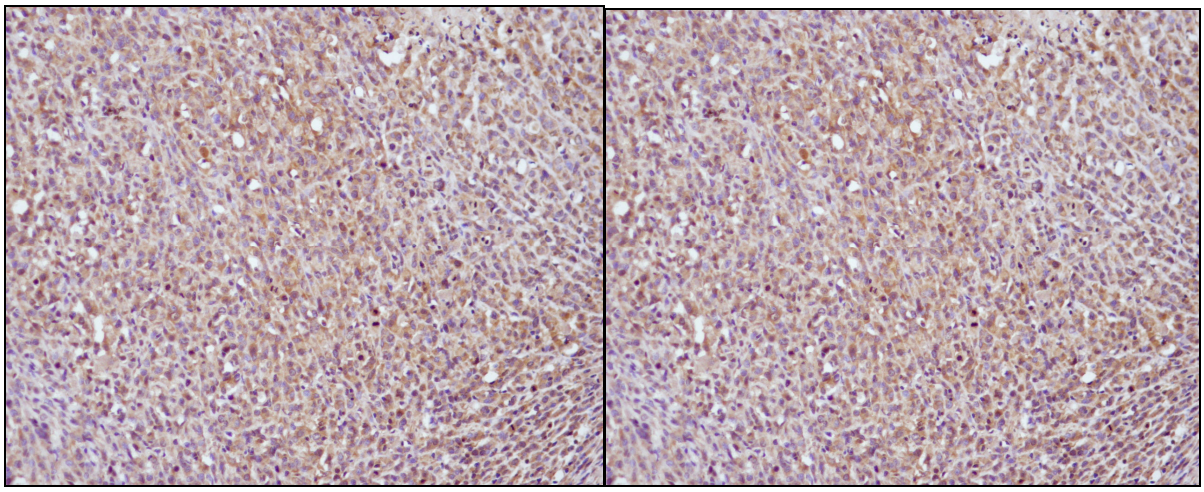
It's evident that the nuclear AhR expression inversely correlated with the cytoplasmic AhR staining.

The same consideration can be made for CYP1A1 expression.

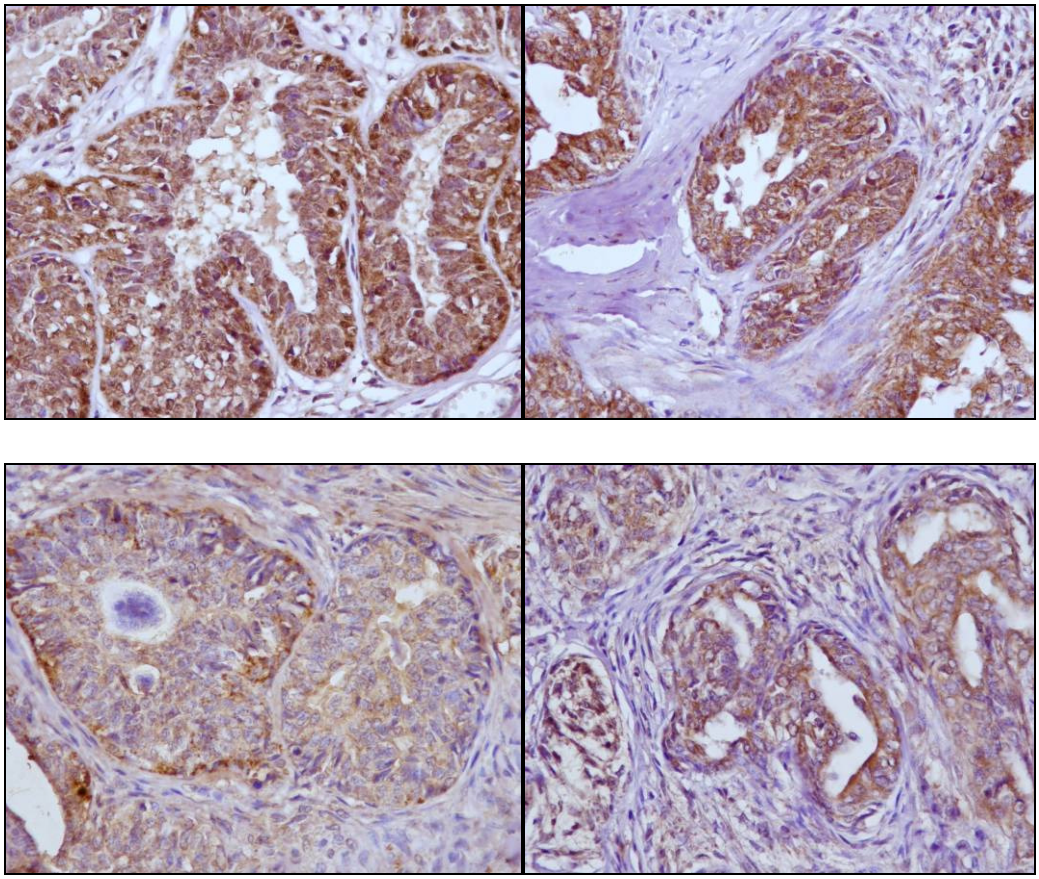
Concerning heavy metals levels; the values show an increase in pathological condition, compare to control samples.(Tab.2)



AhR and CYP1A1 in Lung Cancer



AhR and CYP1A1 in Urinary Bladder Cancer



AhR and CYP1A1 in Epithelial Tumors

Diagnosis	53 Cr	75 As	111 Cd	208 Pb	200 Hg
2-Renal Carcinoma	0,2270	0,0242	0,0065	0,1328	0,032
7-Renal Carcinoma	0,1333	0,0244	0,0060	0,0633	0,018
3-Rectal cancer	0,2084	0,0278	0,0069	0,0937	0,012
9-Prostate cancer	0,4977	0,0192	0,0046	0,0440	0,000
8-Lung Cancer	1,8157	0,0153	0,0129	0,3733	0,000
6-Urothelial cancer	0,2061	0,0129	0,0079	0,0943	0,001
10-Lung Cancer	0,3543	0,0478	0,0124	0,4069	0,101
4-Urothelial Cancer	0,7447	0,0204	0,0046	0,0320	0,091
5-Urothelial Cancer	0,6659	0,0671	0,3069	1,1590	0,035
32-Liver/control	0,0051	0,0046	0,0003	0,0098	0,000
32-Kidney control	0,0064	0,0o32	0,0008	0,0160	0,004

Discussion

Overexpression of the AhR has been detected in all cancerous and pre-cancerous lesions.

It is believed that the nuclear localization of the AhR in such lesions indicates its constitutive activation, although the precise molecular mechanisms leading to such activation remain elusive.

Endogenous AhR that is not activated by exogenous ligand has, in general, pro-proliferative and tumor-promoting properties also .

It's reported a role of the AhR in centrosome duplication control.

Given that supernumerary centrioles can cause cell division errors and chromosomal instability, this finding provides a potential link between the AhR and malignant progression.

Remarkably, overexpression of the AhR can lead to centriole multiplication.

It is noteworthy that centrosome aberrations and multipolar mitoses are frequent findings in pre-cancerous lesions of the mammary gland and the prostate, both tumor entities in which aberrant AhR/CYP1A1 expression has been suggested to play a pathogenic role (30).

We demonstrated that strong nuclear AhR expression was observed in the invasive phenotype and that the nuclear AhR overexpression correlates significantly with malignancy in tumor tissue samples.

The aberrant AhR expression was detected not only in tumor samples but also in a considerable proportion of normal and hyperplastic tissue specimens.

This reflects a role of endogenous AhR in normal cellular functions or a widespread pathological activation of this receptor by one or more ubiquitous xenobiotics.

Concerning the CYP1A1 staining , it's possible to correlate the activation to AhR .

Compare heavy metals levels in tumors and normal control, is evident the increased values in pathological tissue.

The correlation between the heavy metals and cancer must be better investigate.

Conclusions

Actually, it seems very difficult to estimate the real risk for human health correlated with environmental pollution.

Several studies in vitro have suggest the mechanisms and the consequences for exposure to several pollutants but this model can't be used as predictor evaluation. One approach to solving this problem could be to use animals as surrogate monitor or Animal Sentinel.

In our study we use animal tissue to provide an integrated evaluation of heavy metals contamination and AhR- P450 expression during tumorogenesis

P450s are believed to have existed since the beginning of life over 3.5 billion years ago, but the P450s responsible for foreign compound metabolism appear to have arisen about 400 to 500 million years ago. It is believed that these enzymes were needed to metabolise and detoxify chemicals found in plants .

In the plant-animal "warfare" hypothesis, plants produce toxins to kill predators and animals evolve P450s to detoxify these toxins. As this process continues over millions of years new catalytic activities of P450s will develop. However, in the field of chemical carcinogenesis it is difficult to establish a clear relationship in which both the P450s and other foreign compound metabolizing enzymes would evolve for a beneficial purpose.

Although the majority of the studies have focused on the carcinogenic action of CYP1A1, it is recently becoming clear that this enzyme plays important roles in detoxication and chemoprevention, thus opposing the initially established concept, regarding its function in tumor progression.

Moreover, extensive work on the molecular events governing the transcriptional activation of the

CYP1A1 gene through the aryl hydrocarbon receptor has revealed the interplay of AhR with various cell signaling pathways, important in normal cell growth, homeostasis and development. The cross-talk of AhR with different signal transduction pathways is apparent.

However, the precise mechanisms by which AhR ligands elicit toxic responses that may contribute to carcinogenesis still remain unclear. It was previously noted that this may be partially due to the majority of the studies coming from cancer-derived cell lines that have impaired cell cycle regulation and hence do not possess the full detoxication battery.

Studies in non-transformed cells or extrahepatic tissues have been proposed as better models of choice .

Utilization of the animal sentinel systems can unravel the exact mechanisms which regulate the expression of CYP1A1 in tissues, and offer insight into the contribution of the latter in cancer progression or prevention.

If the animals'exposure to environmental pollutants is sufficiently similar to humans'exposure, dogs, as animal sentinel, might provide a reasonable indirect measure of human health condition.

References

1. Griffith J. et al. Cancer mortality in U.S countries with hazardous waste sites and ground water pollution. Arch.Environmental Health 44 (2); 69-74 1989

2. Dolk H. et al. Risk of congenital anomalies near hazardous waste landfill sites in Europe The Eurohazcon study. Lancet 1988, 352(9126); 423-7

3. Musmeci et al. The impact measure of solod waste management on health: The hazard index

Ann Ist Superiore di Sanità 2010,Volume 46. No 3: 293-298.

4. LaDou J et al. 2008. Export of electronics equipment waste. Int J Occup Environ Health 14:1-10.

5. Davis et al. 2010. Opportunities and Constraints for Developing a Sustainable e-Waste Management System at Local Government Level in Australia. Waste Manag Res 28:705-713.

6. Li et al 2007. Severe PCDD/F and PBDD/F pollution in air around an electronic waste dismantling area in China. Environ Sci Technol 41:5641-5646.

7. Ogunseitan O et al.. 2009. Science and regulation. The electronics revolution: from e-wonderland to e-wasteland. Science 326:670-671.

8. Aimin Chen et al. Developmental Neurotoxicants in E-Waste: An Emerging Health Concern Environmental health perspectives. National Institutes of Health U.S. Department of Health and Human Services. Nov 2010.

9. Baccarelli A, Bollati V. 2009. Epigenetics and environmental chemicals. Curr Opin Pediatr 21:243-251.

10. Thomas et al. Genetics of aryl hydrocarbon hydroxilase induction in mice- single gene difference between C57BL/6J and DBA/2J. Biochemical Genet 1972;6 157-168

11. Thomas et al. Genetic relationship between aryl hydrocarbon hydroxilase inducibility and chemical carcinogen induced skin ulceration in mice. Genetics 1973 ;74, 655-659

12. Fan MQ et al. Recombinant expression of aryl hydrocarbon receptor for quantitative ligand binding analyses . Anal. Biochem 2009, 384,279-287

13. Dietrich and Kaina. The aryl hydrocarbon receptor in the regulation of cell-cell contact and tumor growth. Carcinogenesis 2010 Vol. 31 N°8 pp1319-1328

14.Bradshaw TD et al, Relevance of the aryl hydrocarbon receptor for clinical toxicology.Clinical Toxicology (2009) 47, 632-642

15.Villano CM et al TCDD induces MMPs expression and invasion in A"=%(melanoma cells. Toxical Appl. Pharmacol. 2006 310 212 -2214

16. Nelson et al. P450 superfamily: update on new sequences, gene mapping, accession numbers andnomenclature. Pharmacogenetics 1996; 6: 1-42.

17. Capdevila JH et al.Microsomal cytochrome P450 and eicosanoid metabolism. Mol Aspects Med 1999; 20: 42-55

18. Gonzalez FJ, Gelboin HV. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab Rev 1994; 26: 165±183.

19.Windmill KF et al.. The role of xenobiotic metabolizing enzymes in arylamine toxicity and carcinogenesis: functional and localization studies. Mutat Res 1997; 376: 153±160.

20. Fisslthaler B et al Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature 1999; 401: 493-497.

21. Denison MS et al.Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 2003;43:309-34

22. Ikuta T et al. Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. Biochem Biophys Res Commun 2004;317(2):545-50

23. Adachi J. et al. Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. J Biol Chem 2001;276(34):31475-8

24. Seidel SD, Winters GM, Rogers WJ, et al. Activation of the Ah receptor signaling pathway by prostaglandins. J Biochem Mol Toxicol 2001;15(4):187-96

25. VP Androutsopoulos et al., Cytochrome P450 CYP1A1: wider roles in cancer progression and Prevention. BMC Cancer 2009, 9:187

26. Reem H. et al. Transcriptional activation and posttranscriptional modification of Cyp1a1 by arsenite, cadmium, and chromium.. Toxicol Lett. 2007 Aug;172(3):106-19. Epub 2007 May 24.

27. Aposhian, R.A. et al. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species, Toxicol. Appl. Pharmacol. 198 (2004), pp. 327–335.

28. Xu S. et al. Oxidant-mediated AA release from astrocytes involves cPLA(2) and iPLA(2), Free Radic. Biol. Med. 34 (2003), pp. 1531–1543.

29. D.C. Spink et al, Induction of CYP1A1 and CYP1B1 in T-47D human breast cancer cells by benzo[a]pyrene is diminished by arsenite, Drug Metab. Dispos. 30 (2002), pp. 262–269

30. Korzeniewski et al. A novel role of the aryl hydrocarbon receptor (AhR) in centrosome amplification - implications for chemoprevention. Molecular Cancer 2010, 9:153

31. Korashy HM. et al. Regulatory mechanism modulating the expression of cytochrome P450 1A1 gene by heavy metals.