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A B S T R A C T

In humans, the aryl hydrocarbon receptor (AHR) gene battery constitutes a set of contaminant-responsive genes, which have been recently shown to be involved in the regulation of several patho-physiological conditions, including tumorigenesis.

As the domestic dog represents a valuable animal model in comparative oncology, mRNA levels of cytochromes P450 1A1, 1A2 and 1B1 (CYP1A1, 1A2 and 1B1), AHR, AHR nuclear translocator (ARNT), AHR repressor (AHRR, whose partial sequence was here obtained) and cyclooxygenase-2 (COX2) were measured in dog control tissues (liver, skin, mammary gland and bone), in 47 mast cell tumors (MCTs), 32 mammary tumors (MTs), 5 osteosarcoma (OSA) and related surgical margins.

Target genes were constitutively expressed in the dog, confirming the available human data. Furthermore, their pattern of expression in tumor biopsies was comparable to that already described in a variety of human cancers; in particular, both AHR and COX2 genes were up-regulated and positively correlated, while CYP1A1 and CYP1A2 mRNAs were generally poorly expressed.

This work demonstrated for the first time that target mRNAs are expressed in neoplastic tissues of dogs, thereby increasing the knowledge about dog cancer biology and confirming this species as an useful animal model for comparative studies on human oncology.

Introduction

Cytochromes P450 (CYPs) 1A1, 1A2 and 1B1 belong to the CYP superfamily of drug metabolizing enzymes and participate to the bioactivation of environmental pro-carcinogens. The strong elec-trophilic derivatives that are produced are likely to interact with cellular nucleophilic centers, such as DNA, to form adducts; this ultimately triggers mutagenesis and either cell death or neoplastic transformation whenever DNA repair function does not rescue the nucleic acid damage provoked by carcinogen-DNA adducts. Therefore, the constitutive and inducible expression of CYP1A1, CYP1A2 and CYP1B1 are important determinants of carcinogenesis (Oyama et al., 2004).

The transcriptional activation of CYP1 genes occurs through the binding of a ligand (polycyclic aromatic hydrocarbons, aryl and heterocyclic amines) to the aryl hydrocarbon receptor (AHR), a nuclear transcription factor of the basic-helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family of proteins (Nebert et al., 2000; Chang et al., 2003). In normal resting cells, AHR is primarily localized in the cytosol in a complex form. Following the ligand binding to AHR, the receptor translocates to the nucleus, where it binds to the aryl hydrocarbon receptor nuclear translocator (ARNT), another member of the PAS family, as well as to other transcriptional co-activators or co-repressors. Then, the activated AHR complex binds to specific DNA-binding sites localized within promoter regions of target genes (aryl hydrocarbon responsive elements, AHREs) which, in turn, promote the target gene transcription. Recently, a further component of the PAS family, the aryl hydrocarbon receptor repressor (AHRR), has been identified (Yang et al.,

2008). The aryl hydrocarbon receptor repressor is involved in the negative regulation of AHR signaling. Due to high amino acidic similarities with AHR, AHRR may therefore interact with ARNT and bind to AHREs, acting as an AHR competitor. However, the mechanism appears to be more complicated and to involve epige-netic phenomena as well (Mimura et al., 1999).Recent studies have shown that AHR plays also important physiologic roles: it is important for the regulation of autoimmunity

and immune response, cell growth, apoptosis and tumorigenesis (Trombino et al., 2000; Chang et al., 2003; Currier et al., 2005; Funatake et al., 2005; Schlezinger et al., 2006; Vogel et al., 2007; Marshall et al., 2008; Quintana et al., 2008; Hahn et al., 2009; Wong et al., 2009). In fact, AHR is up-regulated in several rodent and human tumor types (Quintana et al., 2008). By contrast, little is known about AHRR gene expression and function in tumors, although it has been hypothesized to play a critical role in cancer as well (Mimura et al., 1999).

Among target genes whose expression is likely to be modulated by AHR itself or AHR ligands such as dioxins, there is the cyclooxy-genase-2 (COX2: Mimura et al., 1999; Vogel et al., 2007; Hahn et al., 2009). Cyclooxygenase-2 is an important inflammatory mediator involved in the conversion of arachidonic acid to prostaglandin endoperoxide, which is then metabolized to various prostanoids by corresponding terminal synthases. In tumors, high COX2 amounts increase angiogenesis, tumor invasion and promotion of tumor cell resistance to apoptosis (Khan et al., 2011). The mechanism by which COX2 inhibit apoptosis is still poorly understood, but high COX2 levels change the ratio of pro-apoptotic and anti-apoptotic proteins toward anti-apoptosis, by reducing the levels of pro-apoptotic and inducing anti-apoptotic protein amounts (Belguise et al., 2007; Khan et al., 2011). Cicloxygenase-2 is also involved in the pathogenesis of several malignancies (Hahn et al., 2009); in addition, an increased COX2 expression is closely related to an enhancement of the pattern of malignant transformation (Lin et al., 2001).

The domestic dog has been used as a model species for screening purposes (i.e., acute, subchronic and chronic toxicity studies) as well as a sentinel species in environmental toxicology studies (Schilling et al., 1988; Hayes et al., 1991; Reif et al., 1992,1995; O'Brien et al., 1993; Gavazza et al., 2001; Hawk et al., 2002; Glick-man et al., 2004). More recently, domestic dog has been shown to be an excellent animal model in comparative oncology (Gavazza et al., 2001; Hawk et al., 2002). Notably, some spontaneous canine tumors (i.e., canine lymphoma and osteosarcoma (OSA)) were in fact comparable with the corresponding human cancer (Reif et al., 1992; Schilling et al., 1988; Santin et al., 2005).

A number of papers regarding the expression of the AHR gene battery and COX2 in tumors have been published; however, these data are from human and rodent model species (Trombino et al., 2000; Hawk et al., 2002; Oyama et al., 2004; Currier et al., 2005; Schlezinger et al., 2006; Vogel et al., 2007). Therefore, in the present study, mRNA levels of AHR, ARNT, AHRR, CYP1A1, CYP1A2, CYP1B1 as well as COX2 were measured in surgical biopsies of mast cell tumors (MCTs), mammary tumors (MTs) and OSA, which represent three of the most commonly occurring canine spontaneous neoplasms. The chosen method of quantification was the quan-titative Real Time RT-PCR (qPCR). The present investigation aimed to confirm whether the pattern of expression of the target genes involved in tumorigenesis is similar between dogs, humans and laboratory species, thereby increasing the knowledge about canine cancer development and confirming the usefulness of the domestic dog for comparative oncology studies.

Materials and methods

Reagents

Chemicals and kits used in this study were obtained from the following companies: chloroform, isopropyl alcohol, and ethyl alcohol (Thermo Electron Corporation, Waltham, MA); TRIzol® reagent and SYBR® Safe gel stain (Invitrogen, Carlsbad, CA); agarose (Sigma Aldrich, Munich, Germany); RNAlater® tissue collection solution and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA); LightCycler® 480 Probes Masterand Universal Probe Library (UPL: Roche Applied Science, Indianapolis, IN); oligonucleotide primers (Eurofins MWG Operon, Ebersberg, Germany).

Clinical and pathological features of tumor patients

Eighty-four tumor biopsies (47 MCT, 32 MT and 5 OSA) were supplied, in a period of about three years, by veterinary clinics located in four cities of Northern Italy (Turin, Mestre-Marghera, Vicenza and Padua). Both male and female dogs were included in the study. The median age registered was 9-10 years (range: 3-16 years old). The following breeds were affected by MCT: Boxer (10 out of 47 cases), Labrador Retriever (7 cases) and crossbred dogs (15 cases). In MTs, crossbred dogs represented 37.5% of cases. Finally, three medium-high canine pure-breeds (Alano, Boxer, Mare-mma Sheepdog) and two crossbred dogs were affected by OSA.

Tumors were classified by three independent pathologists on the basis of diagnostic criteria established for domestic animals tumors classification by the World Health Organization (Misdorp, 2002). Mast cell tumors were further classified according to Patnaik et al. (1984), and consisted of 29 grade 1 (Gl) and 18 grade 2 (G2) samples ; no grade 3 were available. The acronym GO was assigned to surgical margins. Both benign and malignant MTs were considered, and these were conventionally divided into 3 groups according to their histological behavior and instructions reported in the International Classification of Diseases for Oncology (ICD-O), albeit with some modifications: in group 1 (Gl), benign MTs (e.g., adenomas and mixed benign tumors) were included; in G2, complex adenocarcinomas and, in G3, all other carcinomas. Preneoplastic lesions (mammary gland hyperplasia) were grouped as GO. Globally, a total of 15 GO, 9 Gl, 7 G2 and 13 G3 MTs were considered. Finally, 5 OSA, predominantly localized at appendicular sites, were sampled.

A complete list of the tumors enrolled in the study (including the histological diagnosis, dog signaling and sampling location) is reported in the supporting material online (see Supplementary File 1).

Tissue specimens

Samples were collected for both histological and RNA analysis during the surgical intervention. Samples for histology were fixed in 10% neutral buffered formalin and were paraffin-embedded. Then, 4 μm sections were cut and stained with hematoxylin and eosin. For total RNA extraction, 100 mg of each sample was collected and immediately stored in RNAlater® solution at —20 °C until use.

Healthy ("normal") canine tissues of the same anatomical origin of tumor specimens (skin, mammary gland, bone and liver) were used for the measurement of the constitutive expression of target genes in non-neoplastic tissues and their relative quantification (ROJ in pathological specimens. These specimens were obtained from adult, pathogen-free, Beagle dogs offered by GlaxoSmithKline Research center (Verona, Italy) or, alternatively, from adult dogs of different breeds not suffering from neoplasm and euthanized in veterinary clinics of Padua for traumas (i.e., car accidents). Biopsies were collected, in sterility, within 30 min after animal sacrifice and stored in RNAlater® solution at -20 °C until use.

Total RNA isolation from control and tumor samples and reverse transcription

Total RNA was isolated by using the TRIzol® reagent, according to the manufacturer's instructions. Briefly, 1 mL of TRIzol® was added to a small amount of tissue and homogenized twice or three times for 20 s by using 200 mg of silica beads and Ribolyser (Hybaid, Thermo Scientific, Waltham, MA). For bony tissue, the sample was first disrupted in liquid nitrogen with   mortar   and

pestle and, then, suspended in TRIzol® reagent. Samples were then purified with a classical phenol-chloroform extraction step. Total RNA concentration and quality (260/280 and 260/230 nm absorbance ratios) were checked by using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and denaturing gel electrophoresis.

Two micrograms of total RNA and the High Capacity cDNA Reverse Transcription kit were used, according to the manufacturer's procedure, to obtain cDNA, which was then stored at -20 °C until use.

Aryl hydrocarbon receptor repressor sequencing

The canine AHRR mRNA sequence was not available in GenBank and Ensembl Genome Browser databases; therefore, the following approach was undertaken to obtain a partial sequence useful to design the qPCR assay. First, highly conserved regions among nucleotide sequences of different species *{Homo sapiens, Rattus norvegicus, Mus musculus, Bos taurus, Equus caballus, Takifugu rubripes, Danio rerio, Fundulus heroditus, Microgadus tomcod, Xenopus laevis)* were identified on the basis of a multiple alignment approach. On these regions, PCR primer pairs spanning regions of at least 300 bp and partially overlapping were designed and used to amplify canine cDNA. Polymerase chain reactions were carried out by using the GoTaq® Flexi DNA polymerase (Promega, Madison, Wisconsin, USA) in a peqSTAR 96 Universal Gradient thermal cycler (Euroclone, Milan, Italy). After an activation step at 95 °C for 2 min, reactions were continued for 35 cycles (95 °C for 30 s, 57 °C ± 3 °C for 45 s and 72 °C for 90 s). Amplicons were resolved on 1% agarose gel. When a single band of the expected size was obtained, the template was purified by High Pure PCR Cleanup Micro Kit (Roche Diagnostics, Basel, Switzerland) and was directly se-quenced by using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions, on an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA). Unincorporated terminators were removed by isopropanol precipitation. Partial sequences obtained from the sequencing of at least two independent PCR reactions were compared and assembled by means of the software Chromas-Pro 1.5. The canine AHRR mRNA partial sequence of 561 bp was submitted to GenBank [ID GenBank:GU727545] and used for primers and UPL design as described in the quantitative real time PCR (qPCR) section.

Quantitative real time PCR

Canis lupus familiaris mRNA sequences of candidate and reference genes (except for AHRR, whose mRNA sequence was obtained through direct sequencing as described below) were obtained from GenBank and Ensembl Genome Browser web sites (http://ncbi.nlm.nih.gov/ and http://www.ensembl.org/, respectively: see Table 1).

Primers and appropriate probes were chosen by using the UPL Assay Design center web service, a program mainly based on Primer3 software with additional features. Default parameters were used. For each gene, the qPCR assay was chosen among those most highly ranked by the design software. Oligonucleotides were designed to span exon-exon junctions to avoid genomic DNA amplification, and primers sets were subjected to primer test analysis with Oli-goanalyzer Software to exclude self- or ethero - dimers formation. Specific primers sequences and length, amplicon size and the human UPL probe used are shown in Table 1. Two different primer concentrations (300 and 600 nM) and their combinations were checked. A primer combination was considered optimal when the amplification resulted in an amplicon of the expected size and the following conditions were met: a low crossing point

value (where the fluorescence intensity during amplification is significantly greater than the background one), a low standard deviation between replicates, and an adequate signal to noise ratio. The specificity of each qPCR product was evaluated by a 2% agarose gel electrophoresis.

Calibration curves useful to evaluate qPCR performances were obtained after the amplification of decreasing amounts of a cDNA pool diluted at 4-fold intervals. Standard curve analysis of qPCR assays showed high-test linearity (error < 0.2). Amplification efficiencies (E), calculated for each assay from the slope of standard curves, ranged between 1.95 sg E sg 2.03. The dynamic range was generally wider than 10 cycles, except for AHRR, CYP1A1 and CYP1B1 assays (5, 6 and 8 cycles, respectively). The main qPCR parameters (efficiency, linearity and dynamic range) are reported in Table 2.

The canine transmembrane BAX inhibitor motif containing 4 (CGI-119) and Golgin subfamily a 1 (GOLGA1) were chosen as optimal reference genes for the absence of tissue- and pathological state-dependent differences in mRNA expression (Lee et al., 2007). Their amplification efficiency was approximately equal to that of target genes; furthermore, the slight differences in their mRNA levels between normal and pathological samples were not statistically significant.

Polymerase chain reactions were performed on the LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) by using clear LightCycler 480 Multiwell Plates 96 and a final reaction volume of 10 µL. Resulting data were analyzed and quantified with the LightCycler 480 software release 1.5.0 using the second derivative maximum method (Rasmussen, 2001). According to the primer optimization step, specific amounts of forward and reverse primers were mixed in 1 x LightCycler 480 Probes Master containing 100 nmol/L of the selected human UPL probe and 2.5 µL of cDNA as template (about 5 ng). The initial denaturation (95 °C, 10 min) was followed by 45 cycles of 10 s at 95 °C, 30 s at 60 °C, and a final cooling step at 40 °C for 10 s.

The mRNA RQ. was performed by using the ΔΔCt method (Livak and Schmittgen, 2001). Data from control tissues were expressed in RQ. arbitrary units (A.U.), while tumor samples were expressed as fold change compared to the corresponding normal tissue RQ value (skin for MCT, mammary gland for MT and bone for OSA).

Statistical analysis

The statistical analysis of MCT and MT data was performed by using the Kruskal-Wallis test and Dunn's post-test, and the OSA data was compared using an unpaired t-test. The correlation analysis between mRNA levels was performed by using the Spearman non-parametric test. In all circumstances, the Graph Pad Instat 2.01 software (San Diego, California, USA) was used, and a value of $p < 0.05$ was considered as significant.

Results

Canine AHRR mRNA sequence

Using primers designed on highly conserved regions of different species, fragments of partially overlapping canine homologous genes were amplified, sequenced and assembled by means of Chro-masPro 1.5. The sequence was submitted to GenBank [GenBank: GU727545]. The obtained mRNA sequence was finally used to design qPCR primers and probe.

Expression ofAHR gene battery mRNAs in control tissues

The constitutive expression of target genes in healthy tissues is summarized in Fig. 1 and comes from the analysis of at least 3 samples for each tissue (range: 3-7 dogs).

Table 1

Accession number, primers sequence and length, human UPL probes and amplicon size of target and reference genes used in the present study.

Abbreviations: bp, base pairs; F, forward primer; R, reverse primer

Gene acronym	Accession number	Primers sequence (5'–3')	Primers length (bp)	Human UPL probes	Amplicon size (bp)
AHR	[Ensembl:ENSCAFT00000003863]	F: cttcgtgtgccgactaaggt R: tggaaattcattgccagaaa	20 20	120	63
ARNT	[Ensembl:ENSCAFT00000019492]	F: ccacttggacccctagcac R: cttggctgtagcctgagca	19 19	62	68
AHRR	[GenBank:GU727545]	F: attttatgcgtcagcaacaatc R: tgcatcacatccgtctgg	22 18	165	60
CYP1A1	[Ensembl:ENSCAFE00000195626]	F: agggacgttgcgtctttgt R: cgggttaccccatagcttct	19 20	59	65
CYP1A2	[GenBank:NM_001008720.1]	F: tgcagaaaattgtccaggaa R: gagctcttctcattgtgcttca	20 22	136	94
CYP1B1	[Ensembl:ENSCAFE00000009970]	F: gacgccttcatcctctcg R: gcacgtactccatgtccaac	18 20	70	82
COX2	[Ensembl:ENSCANFT00000021841]	F: acctgatgactgtccaacacc R: tccacaatctcttttgaatcagg	21 23	137	72
CGI-119	[GenBank:XM_531662.2]	F: tctacaatctaagagagatttcagcaa R: ttcctgacaagcacaaaatcc	27 21	22	77
GOLGA1	[GenBank:XM_537849.2]	F: ggtggctcaggaagttcaga R: tatacggctgctctcctggt	20 20	149	61

Table 2

Quantitative Real time RT-PCR assays parameters (efficiency, linearity and dynamic range) of target and reference genes used in the present study.

Gene acronym	Efficiency	Error	Dynamic range (Cp)
AHR	1.952	0.001	22.58–34.70
ARNT	2.002	0.005	25.74–37.24
AHRR	2.008	0.027	30.45–35.23
CYP1A1	1.964	0.010	30.13–36.20
CYP1A2	1.989	0.009	26.75–36.73
CYP1B1	2.031	0.005	27.68–35.94
COX2	1.948	0.073	24.23–34.88
CGI-119	1.951	0.008	22.58–34.89
GOLGA-1	1.989	0.007	25.45–36.70

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Canine AHR mRNA was detected in all target tissues, showing the highest level of expression in the liver, followed by skin, mammary gland and, finally, bone. The aryl hydrocarbon receptor nuclear translocator mRNA was ubiquitously expressed and equally distributed. By contrast, AHRR was a rare transcript, with a feeble level of expression in extra-hepatic tissues (skin and mammary gland).

The cytochrome P450 1A1 was constitutively expressed in the liver, but higher mRNA levels were found in skin and mammary gland, where RQ values were about 2.5- and 3-fold greater than the liver, respectively. In contrast, CYP1A2 had the highest expression in the liver (mean RQ= 113.3), but detectable amounts were measured in the skin (RQ=4.7) and mammary gland (RQ=5.5). Both CYP1A isoforms were poorly expressed in the bone. The cytochrome P450 1B1 was mostly expressed in extra-

hepatic tissues, particularly in the bone, where RQ. values were about 20-fold higher than those measured in the liver.

Finally, COX2 mRNA was primarily expressed in the liver (RQof about 7.0) and, to a lower extent, in extra-hepatic tissues (skin>mammary gland>bone, with mean RQ. values of 2.3, 1.0 and 0.5, respectively).

Expression of AHR gene battery mRNAs in tumor specimens

While AHR, ARNT and CYP1B1 were amplifiable and quantifiable in all the tumors, lower or undetectable amounts of AHRR, CYP1A1 and CYP1A2 mRNA were found. Scatter dot plots of global target genes expression profile data, including those referring to tumor biopsies and their respective surgical margins (where available), are reported in the supplemental material online (Supplementary File 2).

Aryl hydrocarbon receptor, AHRR, CYP1A1, CYP1A2, CYP1B1 and COX2 mRNA levels in control tissues, surgical margins and tumor specimens (classified according to the conventional histological grading) are reported in Figs. 2-4. Data are presented in terms of n-fold changes with respect to the corresponding control tissue (K) RQ levels to whom an arbitrary value of 1 was assigned. The only exception is represented by AHRR in OSA, whose mRNA was expressed as RQ, owing to the absence of a detectable mRNA amount in control bone. A more detailed description of results is hereby reported.

Compared to the respective control tissue, AHR mRNA levels were generally increased in tumors (see Fig. 2A-C); about 5-fold in Gl and G2 MCTs ($p < 0.01$ K vs. Gl and K vs. G2, *p* < 0.001 for GO vs. Gl and GO vs. G2, respectively), 4-fold in OSA ($p<0.01$), and only 1.5-2-fold in MTs (not significant, ns). On the contrary, no differences in ARNT gene expression were ever noticed between surgical margins and tumors (see Supplementary File 2). Likewise to AHR, AHRR mRNA was commonly up-regulated in surgical margins and tumors (Fig. 2D-F), but such an increase was never statistically significant.

Among CYPs a collective lower amount of CYP1A1 mRNAs, if compared to K and surgical margins (GO), was found in tumors (see Fig. 3A-C); such a down-regulation was significant for MCTs ($p < 0.05$ K *versus* Gl, $p < 0.01$ K *versus* G2, $p < 0.001$ in GO *versus* Gl and GO *versus* G2; in MTs, the Kruskal-Wallis test was significant ($p = 0.0276$), but the Dunn's post test did not assign a statistical significance to any tested comparison. A similar behavior was noticed for CYP1A2. In tumors, CYP1A2 mRNA levels were usually lower than those measured in K and GO (Fig. 3D-F); in MCTs, significant differences ($p < 0.05$) were found either between GO and Gl than between GO and G2; on the other hand, a p value close to statistical significance (0.0597) was obtained in MTs. As regards CYP1B1, no statistically significant variations were ever noticed in MCTs, although a general increase in gene expression was noticed in tumor biopsies and respective surgical margins (see Fig. 4A-C); on the contrary, higher CYP1B1 mRNA amounts ($p < 0.05$) were noticed in surgical margins and benign tumors (GO and Gl) *versus* G3 in MTs.

A common up-regulation of COX2 mRNA was observed in tumors, reaching a statistical significance in both MCT ($p < 0.05$ K vs. G2, $p < 0.01$ K vs. Gl and GO vs. G2, $p < 0.001$ GO vs. Gl) and OSA ($p<0.05$: Fig. 4D-F); in MTs, the increase of COX2 mRNA did not reach the level of statistical significance ($p = 0.0840$).

Finally, significant correlations between AHR and COX2 mRNAs were noticed in MCT and MT (Spearman r 0.65 and 0.45, p < 0.0001 and p < 0.01, respectively; Fig. 5A-B).

Discussion and conclusions

Spontaneous tumors occurring in domestic animals have recently gained increasing interest within the scientific community and particularly in the field of human cancer biology and transla-tional cancer therapeutics, for their relative high incidence, similar biologic behavior, large body size and comparable responses to cytotoxic agents. Tumor types offering the best comparative interest include lymphoma/leukemia, OSA, melanoma, MTs and colorectal cancer (Vail and MacEwen, 2000; Uva et al., 2009; Tang et al., 2010).

The term AHR gene battery refers to target genes of known carcinogenic environmental pollutants, such as halogenated and polycyclic aromatic hydrocarbons or aryl and heterocyclic amines, which represent known AHR ligands. Basically, the AHR gene battery is involved in the biotransformation (including the bioacti-vation process) of the aforementioned pollutants. Nonetheless, it has been recently demonstrated that AHR, even in the absence of exogenous ligands, plays a role in a number of physiological responses such as cell growth and differentiation, apoptosis, cancer progression and immune response (Mimura et al., 1999); furthermore, AHR itself or its ligands are likely to modulate the expression of different genes, including COX2 (Mimura et al., 1999; Vogel et al., 2007; Hahn et al., 2009). In the present study, mRNA levels of some AHR gene battery members (AHR, AHRR, ARNT, CYP1A1, CYP1A2, CYP1B1) as well as of COX2 were measured in dog tumor biopsies, to clarify whether this set of genes showed a pattern of expression similar to human tumors and would, therefore, confirm the usefulness of the domestic dog as a model species for comparative oncology studies.

We first measured the constitutive expression of target genes in the liver and other extra-hepatic tissues. These latter ones were chosen according to MCT, MT and OSA localization (skin, mammary gland and bone, respectively). The only exception was the liver, that was considered the best reference tissue for CYPs. Both AHR and ARNT were shown to be constitutively expressed in the four dog reference tissues; moreover, AHR had the highest expression in the liver. Altogether, present results agree with rat and human data (Dolwick et al., 1993; Carver et al., 1994; Yamamoto et al., 2004). Canine AHRR was poorly expressed in the skin and mammary glands and was negligible in liver and bone. While liver results substantially confirm data previously reported in rats and humans (Yamamoto et al., 2004; Nishihashi et al., 2006), no information about AHRR gene expression in extra-hepatic tissues is cur-rently available.

As far as AHR target genes is concerned, human CYP1A1 is not constitutively expressed, but it is inducible by AHR ligands in almost all tissues and cell lines (Buters et al., 1999); on the other hand, CYP1A2 is mostly considered as a hepatic CYP isoform in rats (Nishihashi et al., 2006). Likewise to humans, CYP1A1 mRNA was detected in the dog liver, but higher amounts were noticed in the skin and mammary glands; on the other hand, CYP1A2 was predominantly expressed in the liver. The cytochrome P450 1B1 shows a different pattern of expression when compared to CYP1A isoforms; in fact, in humans and mice it is constitutively expressed in steroidogenic tissues, such as the adrenals, ovary, testis, uterus, breast, and prostate (Buters et al., 1999). In the present study, higher CYP1B1 mRNA levels were observed in mammary glands and bone, which are considered steroid-sensitive tissues (Lucas et al., 2007; Carnevale et al., 2010; Clarke and Khosla, 2010). Cyclooxygenase-2 has been

recently included among genes known to be modulated by AHR (Hahn et al., 2009). The human expressed sequence tag profile, available at the National Center for Biotechnology Information link, shows that COX2 is an ubiquitous gene constitutively expressed in the four tissues here considered. To date, similar information were not available for dogs, but present results would support the existing human data. Taken together, it looks like that domestic dogs and humans show a similar pattern of COX2 expression in present target tissues.

Next, mRNA levels of aforementioned genes were measured in MCT, MT and OSA tumor biopsies and surgical margins.

The aryl hydrocarbon receptor mRNA was detected in all tumors and showed a significant up-regulation in MCT and OSA, likewise to other tumor types (Chang et al., 2003). Furthermore, AHR mRNA increased according to tumor invasiveness (the histological grading) in MCTs, supporting data obtained in melanoma cell lines exposed to dioxin (Villano et al., 2006). A similar, albeit not significant, trend was noticed in MTs, and such a behavior may be explained by the fact that these tumors are heterogeneous in terms of cell type, and AHR regulatory effects have been shown to vary according to the cell type (Androutsopoulos et al., 2009). The aryl hydrocarbon receptor nuclear translocator gene expression has been recently correlated with a better prognosis in breast cancer (Martinez et al., 2008). In the present study, ARNT mRNA levels were measured in all tumors without showing significant differences among histological grades. This finding would confirm previous data obtained with melanoma cell lines (Villano et al., 2006). The aryl hydrocarbon receptor repressor inhibits, in several species, both constitutive and xenobiotic-induced AHR transcriptional activity. Whereas the role of AHR in tumorigenesis is well documented (Trombino et al., 2000; Currier et al., 2005; Schlezinger et al., 2006; Yang et al., 2008), few and contrasting information about AHRR expression and function in cancer have been reported (Gradin et al., 1999; Tsuchiya et al., 2003; Mimura et al., 1999; Zudaire et al., 2008). In the present study, AHRR gene expression was not univocal and differed among tumor types, confirming the existing contrasting data. As a consequence, more thorough molecular investigations are needed to clarify the role played by AHRR in carcinogenesis.

In the present study, the AHR up-regulation was not mirrored by CYP1A1,1A2 and 1B1. Basically, several environmental pro-carcinogens, following their binding to AHR, may induce CYP1A and CYP1B, owing to their involvement in the bioactivation process (Nebert et al., 2000; Chang et al., 2003; Oyama et al., 2004). Notwithstanding, both carcinogen-detoxifying and cancer-protecting roles have recently been hypothesized for CYP1A1 (Uno et al., 2004; Androutsopoulos et al., 2009). In general, lower CYP1A1 mRNA amounts were noticed in canine tumors, with significant differences for MCTs and MTs. Such a finding agrees with previous studies made in human breast cancer (El-Rayes et al., 2003; Martinez et al., 2008), mammary cell lines (Chang et al., 2003) as well as in rodent models (dimethyl benzanthracene-induced rat or mouse) of mammary carcinogenesis (Trombino et al., 2000; Quintana et al., 2008). The cytochrome P450 1A2 gene mirrored CYP1A1 behavior, confirming data previously obtained in human prostate and liver cancers (Oyama et al., 2004). As regards CYPIBI, a common trend to up-regulation was recorded in MCTs and OSA, while opposite significant variations were observed in canine MTs, where a higher level of gene expression was observed in surgical margins and benign tumors *versus* malignant specimens. The aryl hydrocarbon receptor and CYPIBI gene expression profiles have been shown to be correlated in breast carcinomas, ovarian tumors and prostate cancers (Tanaka et al., 2002; Listgarten et al., 2004); moreover, AHR preferentially transactivates CYPIBI rather than CYP1A1 in malignant

mammary tumor tissue (Yang et al., 2008). Such an event occurs in the absence of specific AHR ligands, but the causative molecular mechanism has not been clearly identified so far (Chang et al., 2003). On the other hand, conflicting results about CYPIBI gene expression in tumors have also been published (Murray et al., 1997; Shehin et al., 2000; Tanaka et al., 2002; Listgarten et al., 2004; Habano et al., 2009). As a consequence, further studies about molecular mechanisms contributing to tissue- and cancer-specific CYP1B1 gene expression, its relationship with AHR as well as its role in carcinogenesis are needed to draw definitive conclusions.

The aryl hydrocarbon receptor has been recently hypothesized to be involved in the immune response (Mimura et al., 1999; Haar-mann-Stemman et al., 2009); in fact, dioxin exposure increased the transcription of CAAT/enhancer-binding protein p, a key transacti-vator of COX2 expression (Vogel et al., 2007; Hahn et al., 2009). Furthermore, AHR activation in lymphoma and leukemia was associated with a clear increase of COX2 gene expression (Hahn et al., 2009). Thus, a link between AHR and COX2 activation was demonstrated. In the present study, the overall increase of AHR and COX2 mRNA levels and the resulting significant correlation found in MCT and MT confirm abovementioned data (Vogel et al., 2007; Hahn et al., 2009). Likewise to AHR, COX2 gene expression increased with MCT and MT aggressiveness confirming previous data obtained through a microarray approach in human breast cancer (Wulfing et al., 2003).

In conclusion, this is one of the few papers (if any) published in comparative oncology and investigating the AHR gene battery expression in dog control tissues, tumor biopsies and surgical margins obtained *ex vivo.* In fact, most studies come from human or laboratory species cancer-derived cell lines, showing some limitations (e.g., an impaired cell cycle regulation and a lower, if any, expression and activity of drug-metabolizing enzymes) and, thereby, circumventing to clearly characterize molecular mechanisms by which AHR contributes to carcinogenesis. Present results demonstrate that members of AHR gene battery as well as COX2 are expressed to a varying extent in three of the most common canine tumors and their respective control tissues. Their pattern of expression was in general comparable with data previously reported for rat and human cancers; interestingly, the grading-dependent transcriptional modulation observed for AHR and COX2 (up-regulation) and CYP1A1/2 (down-regulation) might probably depend essentially on the pathological state (cancer) rather than an hypothetical exposure to environmental pollutants. Therefore, it is conceivable to hypothesize that AHR gene battery and COX2 are likely to be involved in tumorigenesis in dogs, likewise to human and rodent model species. Taken together, present results increase the knowledge about dog cancer biology and further confirm this species as an useful animal model for comparative studies on human cancer biology. Clearly, further in-depth molecular studies are needed to better clarify the role played by these target genes in different canine tumors.
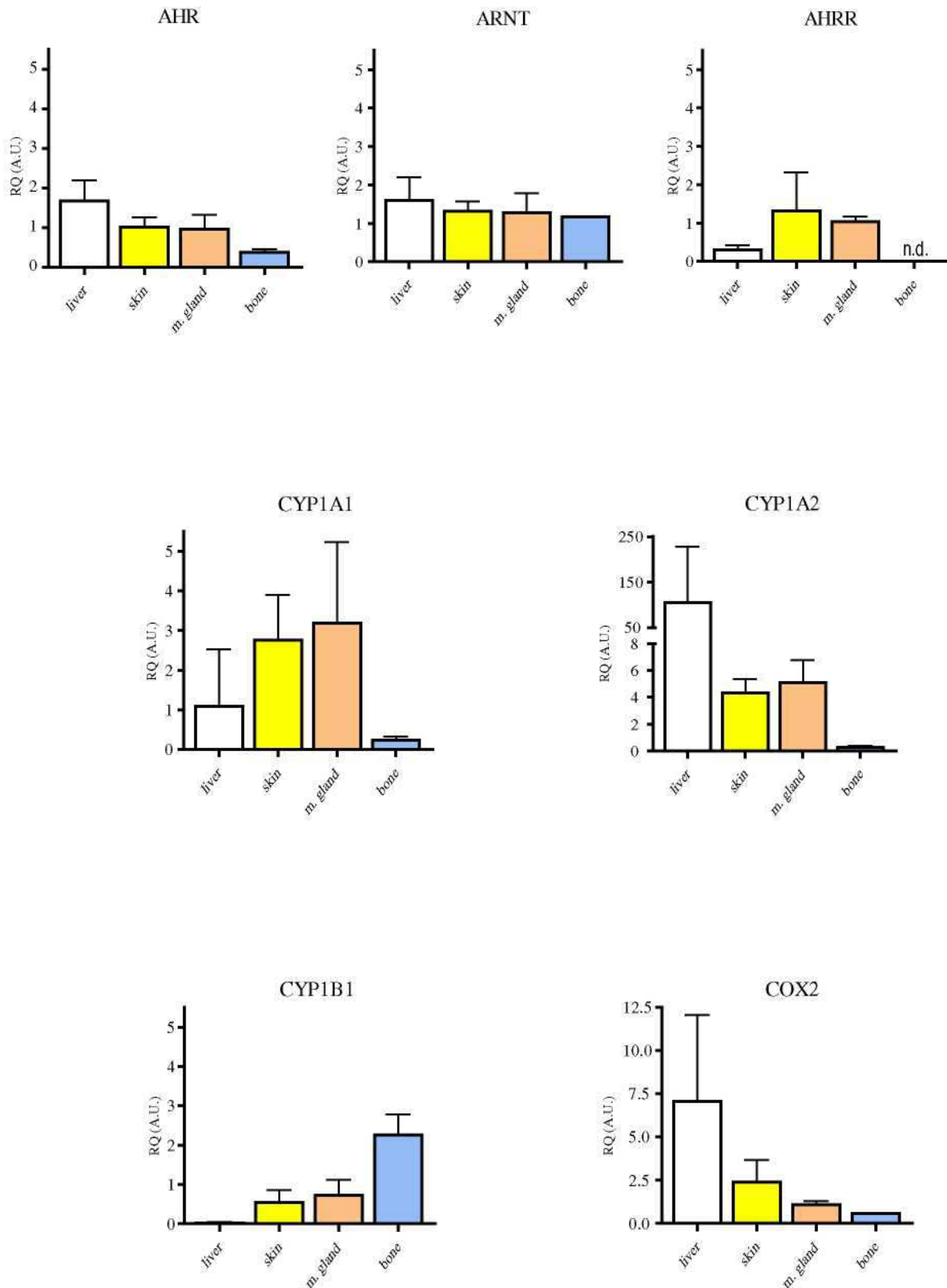
Fig. 1. Expression of target genes (AHR, ARNT, AHRR, CYP1A1, CYP1A2, CYP1B1, and COX2) in control tissues. The mRNA levels were measured in liver, skin, mammary gland and bone specimens obtained either from Beagles or adult dogs euthanized for poor general conditions or traumas. Data (means ±SD) are expressed in RQ. values (A.U.) obtained by using the AACt method.
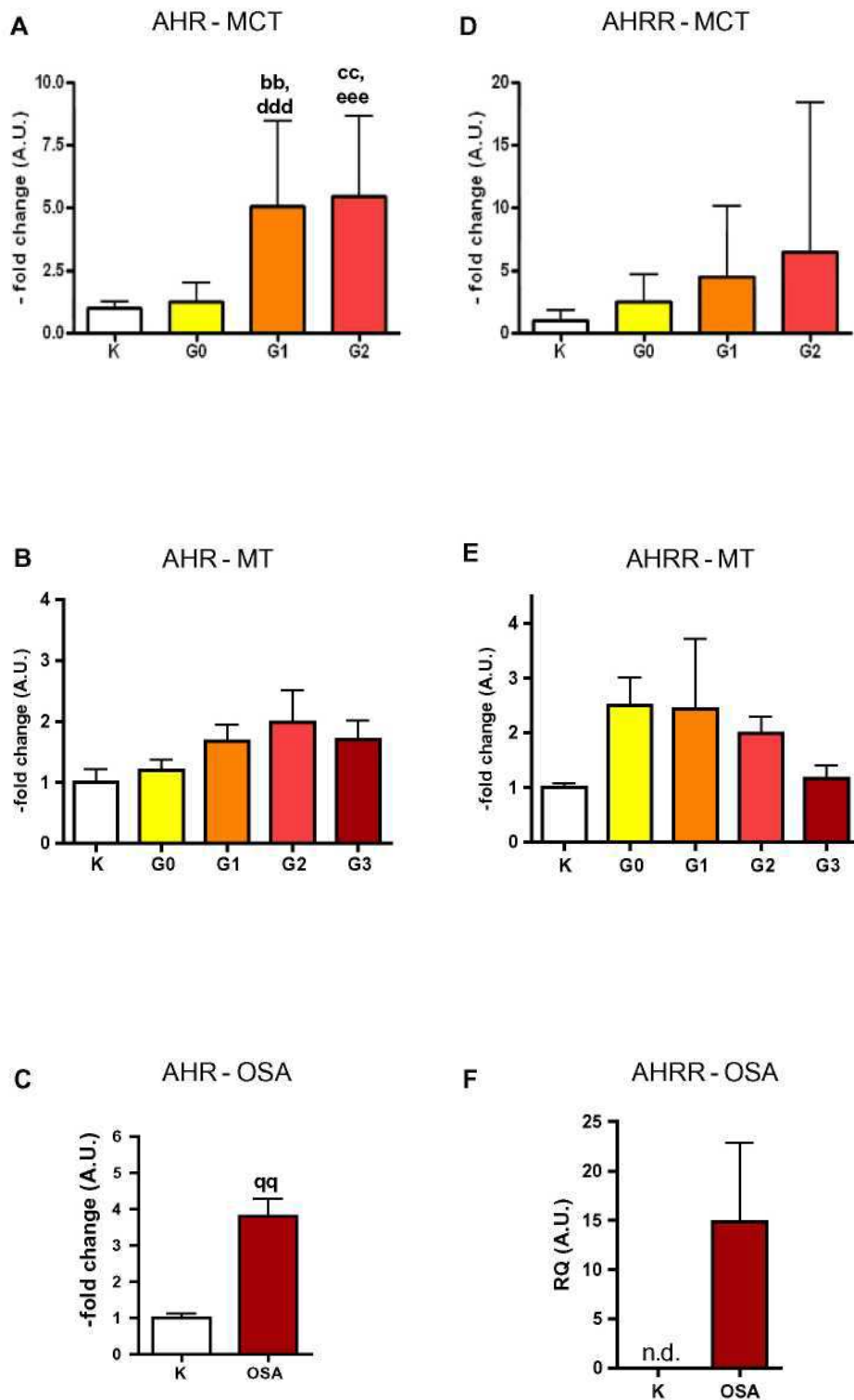
Fig. 2. Expression of AHR and AHRR mRNAs in tumor specimens. Amounts of AHR and AHRR mRNAs were measured in MCTs (A, D), MTs (B, E), OSA (C, F) and in their respective surgical margins. Data were expressed as n-fold changes (means ± SD, A.U.) obtained through the normalization of AACt RQ. values to those of the respective control tissue, to whom an arbitrary value of 1 was assigned. Statistical analysis: Kruskal-Wallis followed by Dunn's post test; unpaired t-test. b: K vs Gl; c: K vs G2; d: GO vs Gl; e: GO vs G2; q: K vs OSA. bb,cc,qq: $p < 0.01$; ddd,eee: $p < 0.001$. K, control tissue; GO, surgical margins; Gl, G2, G3, histological grading.
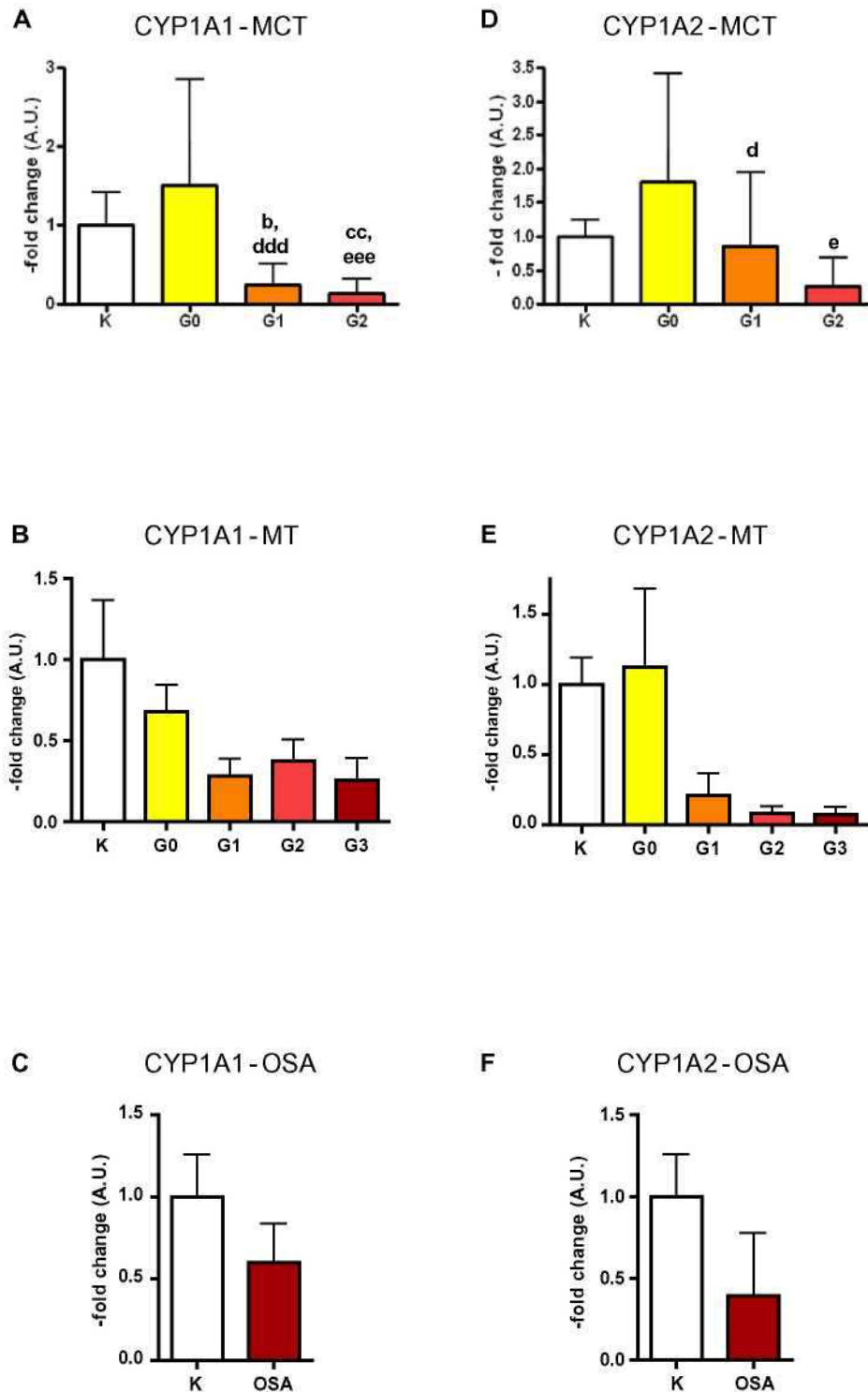
Fig. 3. Expression of CYP1 Al and CYP1A2 mRNAs in tumor specimens. Amounts of CYP1A1 and CYP1A2 mRNA were measured in MCTs (A, D), MTs (B, E), and OSA (C, F) and in their respective surgical margins. Data were expressed as n-fold changes (means ± SD, A.U.) obtained through the normalization of AACt RQ. values to those of the respective control tissue, to whom an arbitrary value of 1 was assigned. Statistical analysis: Kruskal-Wallis followed by Dunn's post test, b: K vs Gl; c: K vs G2; d: GO vs Gl; e: GO vs G2. b.d.e. p< 0.05; [cc]: p < 0.01; [dddeee]: p < 0.001. K, control tissue; GO, surgical margins; Gl, G2, G3, histological grading.
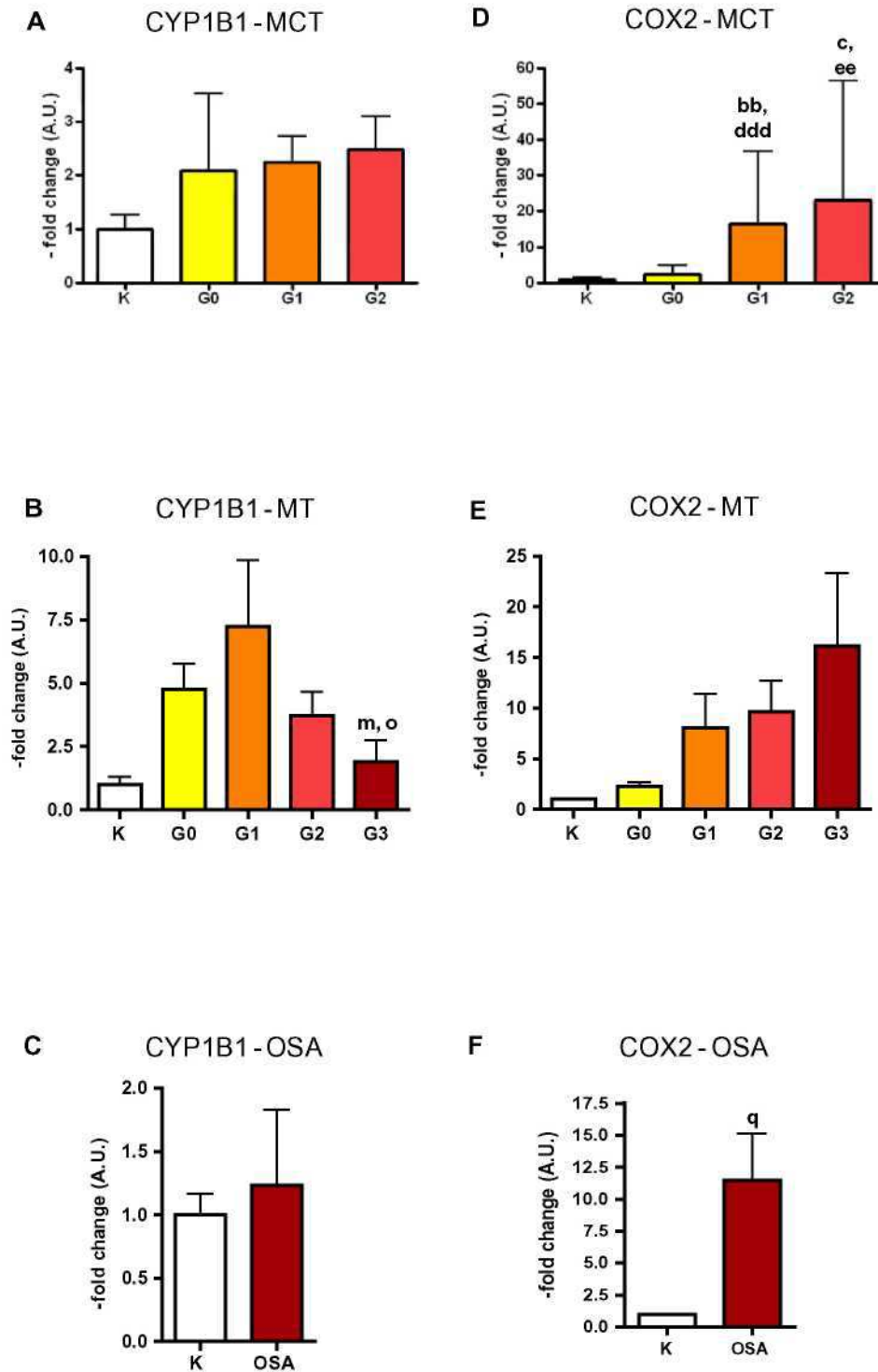
Fig. 4. Expression of CYPIBI and COX2 mRNAs in tumor specimens. Amounts of CYPIBI and COX2 mRNA were measured in MCTs (A, D), MTs (B, E), OSA (C, F) and in their respective surgical margins. Data were expressed as n-fold changes (means ± SD, A.U.) obtained through the normalization of AACt RO_ values to those of the respective control tissue, to whom an arbitrary value of 1 was assigned. Statistical analysis: Kruskal-Wallis followed by Dunn's post test; unpaired t-test. b: K vs Gl; c: K vs G2; d: GO vs Gl; e: GO vs G2; m: GO vs G3; o: Gl vs G3; q: K vs OSA.[cmotl]: $p < 0.05$; [bbee]: $p < 0.01$; [ddd]: $p < 0.001$. K, control tissue; GO, surgical margins; Gl, G2, G3, histological grading.
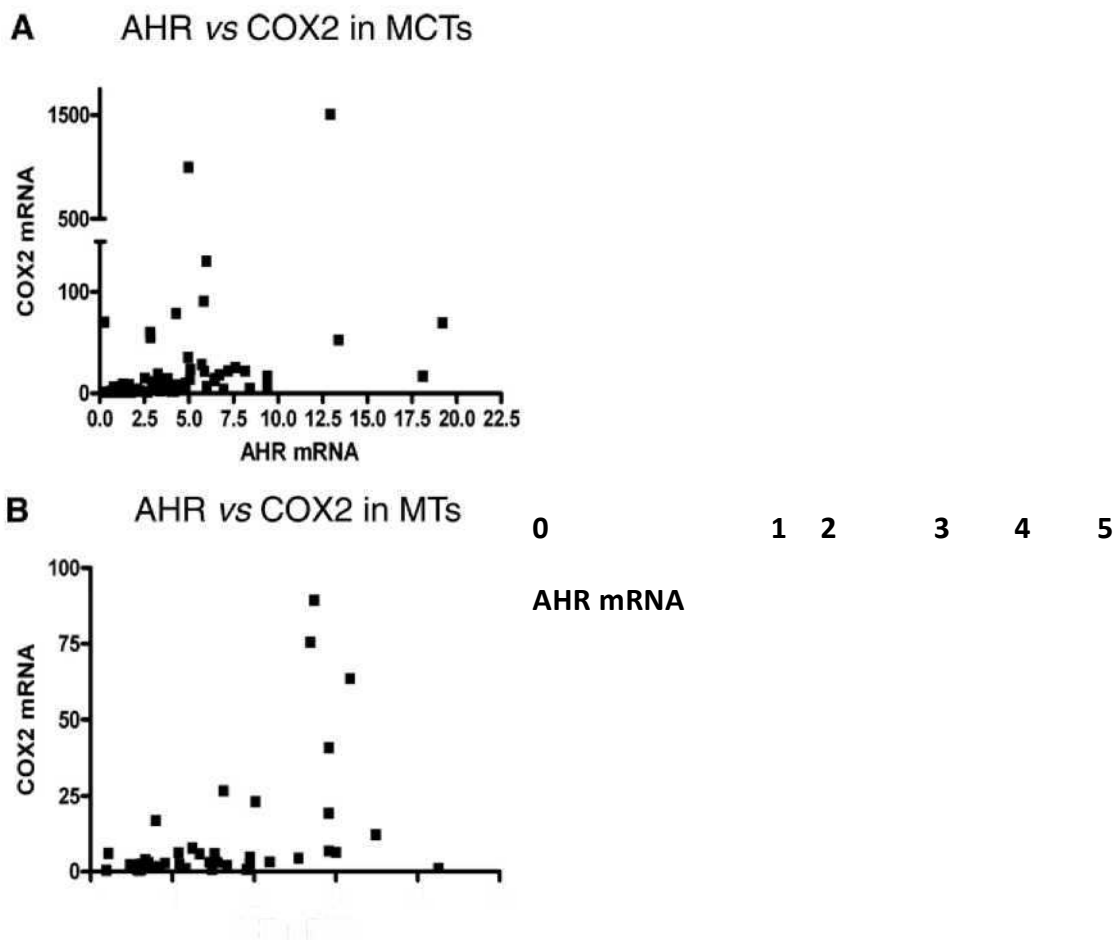
Fig. 5. Spearman correlation analysis between AHR and COX2 mRNAs in MCTs (A) and MTs (B).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.Org/10.1016/j.rvsc.2012.07.035.

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