UNIVERSITY OF VERONA

LIFE SCIENCES AND REPRODUCTION DEPARTMENT

SECTION OF BIOLOGY AND GENETICS

PhD SCHOOL IN

TRANSLATIONAL BIOMEDICINE

CYCLE XXIII

MUTATION SEARCH AND ASSOCIATION STUDY OF CANDIDATE GENES IN NON MELANOMA SKIN CANCER AFTER ORGAN TRANSPLANTATION

MED/03

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CONTENTS

ITALIAN ABSTRACT

La Prostaglandina E2 (PGE2) è coinvolta in diversi processi fisiopatologici. PGE2 è una molecola chiave nel processo di cancerogenesi, essendo coinvolta nella proliferazione cellulare, nell'angiogenesi, nella sorveglianza immunitaria e nell'apoptosi.

La sintesi di PGE2 richiede tre enzimi che agiscono in sequenza: la Fosfolipasi A2, la cicloossigenasi e la sintasi della prostaglandina E. La Fosfolipasi A2 (PLA2) permette il rilascio di acido arachidonico (AA) dai fosfolipidi di membrana, la cicloossigenasi (COX) converte l'AA a PGH2, che viene isomerizzato a PGE2 dalla sintasi della prostaglandina E (PGES). PGE2 viene rilasciato dalle cellule e interagisce con quattro distinti recettori, EP1, EP2, EP3 e EP4.

Una sovra-espressione della forma inducibile di COX (Cox-2) e di PGES microsomiale-1 (mPGES-1), con conseguente eccessiva produzione di PGE2, è stata osservata in vari tessuti tumorali, tra cui alcuni tumori cutanei. Una sovra-espressione di EP1 è stata osservata in tumori cutanei indotti da UVB. Varianti genetiche, situate nella regione del promotore dei geni codificanti per la prostaglandina sintasi-2 (PTGS2/COX2), PGES microsomiale-1 (mPGES-1), o per il recettore EP1 (PTGER1), potrebbero determinare alterazioni nella espressione genica, con una conseguente aumentata sintesi di PGE2 o alterata risposta a PGE2. Tali varianti potrebbero pertanto rappresentare fattori di rischio per lo sviluppo di tumore cutaneo non melanoma (non melanoma skin cancer NMSC) in individui riceventi trapianto d'organo (OTRs).

Per determinare se polimorfismi in questi geni potessero costituire utili marcatori genetici di suscettibilità, contribuendo alla individuazione precoce di individui a maggiore rischio di NMSC, è stato eseguito uno studio caso-controllo. Due polimorfismi nel gene PTGS2, tre nel gene mPGES-1 e tre nel gene PTGER1 sono stati genotipizzati in 286 OTRs, 144 casi NMSC e 142 controlli.

L'allele –765G del gene PTGS2 è significativamente più frequente nei casi rispetto ai controlli [p=0.015; OR=9.59 (1.36-192.66)], suggerendo che questa variante possa rappresentare un fattore di rischio per lo sviluppo di tumori basocellulari (BCC) nei soggetti sottoposti a trapianto prima dei 50 anni.

L'analisi dei polimorfismi –1760C>A (rs3810254), -1728G>A (rs3810255) e –1113C>T (rs2241359) della regione 5' prossimale del gene PTGER1 ha dimostrato che gli alleli

minori per queste varianti erano più rappresentati in individui con tumore squamocellulare (SCC), rispetto ai corrispondenti controlli sottoposti a trapianto prima dei 50 anni, ma la differenza non è risultata statisticamente significativa. Per verificare se una regione di 1452 pb del 5' fiancheggiante il gene, e contenente i tre polimorfismi, esercitasse un'attività di promotore, è stata eseguita l'analisi funzionale in colture cellulari HeLa e HaCat (cheratinociti umani). Nonostante una debole attività di promotore sia stata osservata in cellule HeLa, non siamo stati in grado di dimostrare attività di promotore nelle cellule HaCat, anche dopo stimolazione con LPS.

La regione del 5' fiancheggiante il gene mPGES-1 è stata analizzata mediante l'analisi degli eteroduplici per identificare nuove varianti. Tre polimorfismi, situati all'interno di regioni conservate del gene e riportati nel database NCBI come –664T>A (rs7873087), –663A>T (rs7859349) e –439T>C (rs7872802), sono stati identificati. Le distribuzioni genotipiche osservate hanno indicato completo linkage disequilibrium per i tre polimorfismi, e nessuna associazione con NMSC.

In conclusione, l'allele -765C nel gene PTGS2 sembra rappresentare un fattore protettivo verso lo sviluppo di BCC negli individui sottoposti a trapianto prima dei 50 anni. L'analisi dei polimorfismi nella regione 5' fiancheggiante i geni mPGES-1 e PTGER1 tende ad escludere l'ipotesi che varianti in queste regioni possano rappresentare fattori di rischio per la predisposizione a NMSC.

ENGLISH ABSTRACT

Prostaglandin E2 (PGE2) is a prostanoid with a variety of bioactivities, and has been implicated in various pathologies. PGE2 appears as a key molecule in tumour formation, involved in cell proliferation, angiogenesis, immune surveillance, and apoptosis.

PGE2 is produced via three sequential enzymatic reactions: release of arachidonic acid (AA) from membrane glycerophospholipids by phospholipase A2 (PLA2), conversion of AA to the unstable intermediate prostanoid PGH2 by cyclooxygenase (COX), and isomerization of PGH2 to PGE2 by prostaglandin E synthase (PGES). PGE2 is released from cells and it interacts with four distinct receptors, EP1, EP2, EP3, and EP4.

Over-expression of the inducible form of COX (Cox-2) and microsomal-prostaglandin E synthase-1 (mPGES-1), resulting in excessive prostaglandin E 2 (PGE2) production, has been observed in cancer of various tissues, including skin cancer. An over-expression of EP1 has been observed in skin cancer development induced by UVB. Alteration in gene expression, due to genetic variants located in the promoter region of the genes for prostaglandin synthase-2 (PTGS2/COX2), microsomal prostaglandin E synthase (mPGES-1), or prostaglandin E receptor 1 (PTGER1) may result in augmented PGE2 synthesis or in altered response to PGE2, and could represent risk factors for the development of non melanoma skin cancer (NMSC) in organ transplant recipients (OTRs).

To determine if polymorphisms in these genes can be useful genetic markers of susceptibility that may contribute to early detection of individuals at greater risk of NMSC, a case-control study was performed. Two polymorphisms in the PTGS2 gene, three in the mPGES-1 gene, and three in the PTGER1 gene were genotyped in 286 OTRs, 144 NMSC cases and 142 controls.

Allele –765G in the PTGS2 gene was more frequent in cases than in controls [p=0.015, OR=9.59 (1.36-192.66)], suggesting that this variant might represented a risk factor in the development of basal cell cancer (BCC) in individuals undergoing transplantation before 50 years of age.

Analysis of polymorphisms –1760C>A (rs3810254), -1728G>A (rs3810255), and –1113C>T (rs2241359) in the 5’ proximal region of the PTGER1 gene showed that minor alleles of these variants were more represented in individuals with squamous cell cancer

(SCC), when compared to matched controls who underwent transplantation before 50 years of age, but the difference did not reach significance. To verify if the 1452 bp fragment of the 5' flanking region, which contains the three polymorphisms could exert promoter activity, we performed functional analysis in HeLa and HaCat cultured cells. Although a weak promoter activity was observed in HeLa cells, we were unable to demonstrate any promoter activity in HaCat cells, even after LPS stimulation.

The 5' flanking region of the mPGES-1 gene was screened by heteroduplex analysis to identify new variants. Three polymorphisms, located within conserved regions of the gene, and reported in NCBI databases as –664T>A (rs7873087), –663A>T (rs7859349) and –439T>C (rs7872802), were identified. The observed genotype distributions indicated complete linkage disequilibrium for the three polymorphisms, and no association with NMSC was observed.

In conclusion, allele –765C in the PTGS2 gene seems to represent a protection factor against the development of BCC tumours in individuals undergoing transplantation before 50 years of age. Analysis of polymorphisms in the 5' regions of the mPGES-1 and PTGER1 genes did not support the hypothesis that variants in these regions could play a major role in NMSC predisposition.

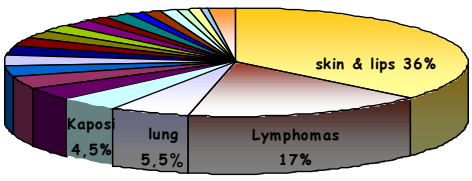
INTRODUCTION

Organ transplant recipients (OTR) are at increased risk of developing both systemic and cutaneous cancers, with an overall increased risk 3- to 4-fold greater than that in the general population (Sheil, 1992). The most common types of post-transplantation cancers include skin cancer, post-transplantation lymphoma, cervical carcinoma, and Kaposi's and other types of sarcomas (Penn, 2000a; Penn, 2000b; First and Peddi, 1998) (**Tab. 1** and **Fig. 1**).

Other epithelial malignancies, including urogenital tract, gastrointestinal tract, laryngeal, and bronchial carcinomas, occur at an increased rate, in contrast to the rates of cancer prevalent in the general population, such as breast and prostate cancers, which are not increased in transplant recipients (Penn and Brunson, 1988).

Type of neoplasm	% of tumors
Cancer of skin and lips	36.3
Lymphomas	17.1
Carcinoma of lung	5.5
Kaposi sarcoma	4.1
Carcinoma of the uterus (cervix 313, body 54, unspecified 3)	4.0
Carcinoma of the colon and rectum	3.5
Carcinoma of the kidney (native kidney 278, allograft 31, unspecified 13)	3.5
Carcinoma of the breast	3.1
Carcinoma of the head and neck (excluding thyroid, parathyroid, and eye)	2.9
Carcinoma of the vulva, perineum, penis, and scrotum	2.5
Carcinoma of the urinary bladder	2.2
Metastatic carcinoma with primary site unknown	2.2
Leukemias	1.9
Carcinoma of the prostate gland	1.7
Hepatobiliary carcinomas	1.7
Sarcomas (excluding Kaposi sarcoma)	1.2
Carcinoma of the thyroid gland	1.2
Carcinoma of the stomach	1.2
Testicular carcinoma	0.8
Carcinoma of the pancreas	0.8
Ovarian cancers	(0.7
Miscellaneous tumors	1.9
Total	100

Tab. 1 and Fig. 1: De Novo Tumours in Organ Transplant Recipients Reported to the Cincinnati Transplant Tumour Registry (First and Peddi 1998)



Frequencies of tumors after transplantation

1. Skin cancers

Skin cancers are the most frequent malignancies after organ transplantation (Euvrard et al., 2003; Ulrich et al., 2004), with 95% of them being non melanoma skin cancers (NMSC) (**Fig. 2**), especially squamous cell carcinomas (SCC) and basal cell carcinomas (BCC). The incidence of some rare tumours (such as melanoma, Merkel cell Carcinoma and Kaposi's sarcoma) is also increased in this group of patients compared with the general population (Dreno, 2003; Zafar et al., 2008).

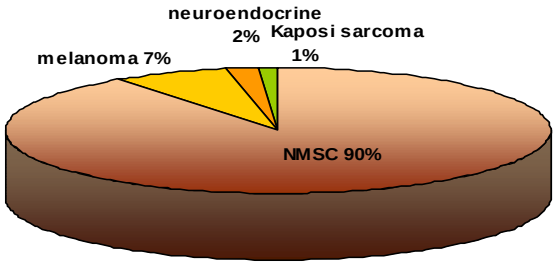


Fig. 2: Frequency and type of skin cancer occurring in OTR patients

Malignant melanoma: is a type of cancer arising from the melanocyte cells of the skin. Although transplant recipients appear to be at lower risk for melanoma than for other skin cancers, the risk has been reported to be increased by a factor of 1.6 to 3.4 in Europe (Jensen et al., 1999; Birkeland et al.,1995;) and by a factor of 2 to 4 in Australia, as compared with the risk in the general population (Bouwes-Bavinck et al., 1996 ; Sheil et al., 1987).

Kaposi's sarcoma: the incidence is much higher in transplant recipients than in non immunosuppressed people (by a factor of 84 to 500) (Euvrard et al., 2003). This cancer usually starts within the dermis but can also form in internal organs. Cutaneous sarcomas other than Kaposi's sarcoma have been reported in transplant recipients (Penn, 1995). However, the small number of reported cases prevents conclusions about their incidence and whether it is higher in such patients than in the general population. These tumours include angiosarcomas, fibroxanthomas, leiomyosarcomas, and dermatofibrosarcoma protuberans.

Merkel cell carcinoma: this rare type of skin cancer develops from neuroendocrine cells (hormone-making cells that resemble nerve cells in some ways) in the skin. These cancers are thought to be caused in part by sunlight exposure. The lesion develops an average of 7.5 years after transplantation, and is usually located on the head or neck and on the arms (Euvrard et al., 2003).

Skin lymphomas: they affect up to 5 percent of all transplant recipients. Seventy percent of cutaneous lymphomas are B-cell lymphomas in origin, presenting as single or multiple papules or nodules on the face, trunk, or limbs, which are occasionally ulcerated. The other 30 percent of cutaneous lymphomas are T-cell lymphomas in origin, manifested clinically as mycosis fungoides, erythroderma, or hemorrhagic lesions, usually with generalized lymphadenopathy (Euvrard et al., 1992).

Keratoacanthomas: these are growths that are found on sun-exposed skin. Although they may start out growing quickly, their growth usually slows down. Many keratoacanthomas shrink or even go away on their own over time without any treatment. But some continue to grow, and a few may even spread to other parts of the body. Because their growth is often hard to predict, many skin specialists think it is safest to consider them as a form of squamous cell skin cancer.

1.1 Non melanoma skin cancer

Non melanoma skin cancers affect cells different from melanocytes: keratinocyte cells, fibroblasts, endothelial cells, although usually NMSC refers to basal cell carcinomas (BCC) and squamous cell carcinomas (SCC).

The first sign of BCC is often a small red, pink or pearly lump, which appears on previously normal skin (**Fig. 3a**). The lump is often dome-shaped. However, BCCs can vary in shape and colour. They usually grow very slowly.

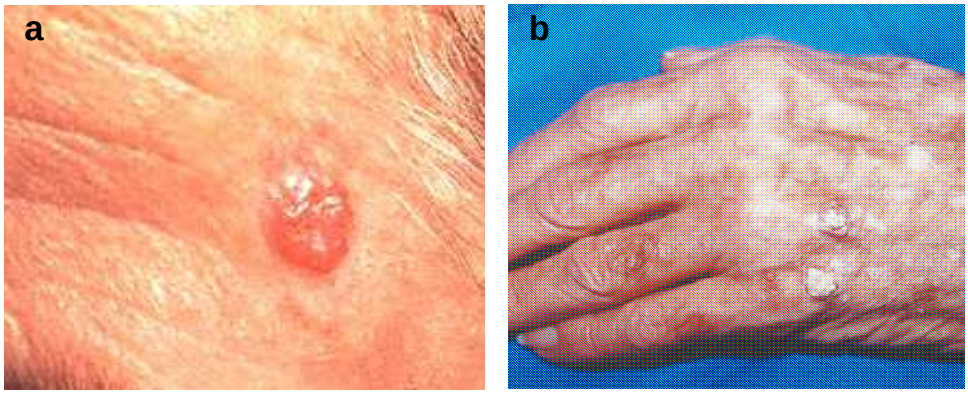


Fig. 3: Examples of Basal Cell Carcinoma (BCC) (**a**) and Squamous Cell Carcinoma (SCC) (**b**)

SCC typically starts as a small crusted or scaly area or skin with a red or pink base (**Fig. 3b**). It may grow into a lump which may look like a wart. An SCC may ulcerate or bleed from time to time. However, an early SCC can vary in shape, appearance and colour. Squamous cell carcinoma in situ, also called Bowen disease, is the earliest form of squamous cell skin cancer. "In situ" means that the cells of these cancers are still entirely within the epidermis and have not yet spread to the dermis.

The incidence of NMSC increases steadily with time after transplantation, and varies in the United States and Western Europe from 5% to 10–27% to 40–60% at 2, 10 and 20 years, respectively (Kasiske et al., 2004; Ramsay et al., 2007; Bordea et al., 2004).

Several studies have shown a 2- to 4-fold higher incidence in heart transplant recipients (HTR) as compared with kidney transplant recipients (KTR) (Ulrich et al., 2004; Fortina et al., 2000; Otley et al., 2005). Studies on skin cancers in liver transplant recipients (LTR) are more numerous (Ulrich et al., 2004; Euvrard et al., 2006); current data suggest that their incidence could be similar to KTR (Otley et al., 2005; Euvrard et al., 2006).

In OTR the ratio of SCC to BCC (4/1) is reversed compared with the population at large, and this reversal increases with decreasing latitude, sunlight exposure, and length of follow-up (Euvrard et al., 2003; Ulrich et al., 2004). While age-matched KTR and HTR show similar ratios, LTR seem to have a higher rate of BCC.

2. Risk factors in NMSC

2.1 UV radiation and skin cancer

Ultraviolet (UV) radiation in sunlight is the most prominent and ubiquitous known physical carcinogen in our natural environment. It is highly genotoxic but does not penetrate the body any deeper than the skin. The shorter wavelengths of this spectrum, UVB (290–320 nm) is absorbed within the epidermis and upper dermis and causes erythema and sunburns (**Fig. 4**).

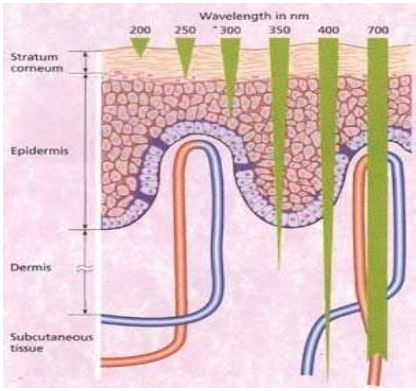


Fig. 4: UV wavelength and skin

Like all organisms regularly exposed to sunlight, the human skin is extremely well adapted to continuous UV stress. Well-pigmented skin is clearly better protected than white Caucasian skin. The sun-seeking habits of white Caucasians in developed

countries are likely to have contributed strongly to the increase in skin cancer observed over the last century. Skin cancer is by far the most common type of cancer in the U.S.A. and Australia, which appears to be the result of an unnatural displacement of people with sun-sensitive skin to sub-tropical regions. Although campaigns have been successful in informing people about the risks of sunlight exposure, general attitudes and behaviour do not yet appear to have changed to the extent that trends in skin cancer morbidity, and the corresponding burden on public healthcare, will be reversed. The relationship between skin cancer and regular sun exposure was suspected by physicians in the late 19th century, and subsequently substantiated in animal experiments in the early part of the 20th century. UV radiation was found to be highly genotoxic, and DNA repair mechanisms proved to be crucial in fending off detrimental effects such as mutagenesis and cell death. In fact, around 1940 it was shown that the wavelength dependence of mutagenicity paralleled the UV absorption by DNA. In the 1970s research on UV carcinogenesis received a new impetus from the arising concern about a possible future depletion of the stratospheric ozone layer: the resulting increases in environmental UV loads were expected to raise skin cancer incidences. Epidemiological studies in the last decades of the 20th century have greatly refined our knowledge on the aetiology of skin cancers. Molecular analyses of gene mutations in skin carcinomas have identified UV radiation as the cause (de Gruijl et al., 2001).

UV radiation induces redox imbalance affecting cell membrane, cell cycle, and the rate of apoptosis in both human melanocytes and keratinocytes (Larsson et al., 2005).

UVA is absorbed within the skin and gives rise to photosensitizing reactions by interaction with endogenous chromophores. In these reactions, singlet oxygen is initially formed, followed by other reactive oxygen species. Singlet oxygen alters signal transduction within a cell, and various studies have demonstrated the activation of several genes following this “trigger”, including oxygenase-1 (Basu-Modak and Tyrrell, 1993), NF-κB (Vile et al., 1995a), and STAT1 (Vile and Tyrrell, 1995b). Convincing evidence has been presented for singlet oxygen-mediated oxidation of cholesterol, lipids, DNA, and proteins in cell culture models (Girotti, 2001; Wright et al., 2003; Phillipson et al., 2002). Such reactions lead to impaired cellular functions, and the reaction products might contribute to DNA damage and increased risk of malignant transformation. For example, UVA appear to induce genomic instability in immortalized human keratinocyte cells (HaCat) by an oxidative stress mechanism (Phillipson et al., 2002). Moreover, several reports have documented beneficial effect of antioxidants in protecting skin cells against UV irradiation, as well as endogenous protection by subsequent induction of manganese superoxide

dismutase and glutathione (GSH) peroxidase in human dermal fibroblasts (Meewes et al., 2001). UVB caused no immediate detectable oxidative damage in normal human melanocytes (MCs); however, a significant effect on cell viability can be observed 24 h after irradiation. Further, the rate of apoptosis appears increased after UVB exposure which can be an indication of a consequence of direct DNA damage.

2.2 Other factors that increase risk of NMSC development

Genetic predisposition and nonenvironmental factors such as skin type can exacerbate the risk posed by solar radiation. Fair-skinned individuals who have received high levels of sunlight exposure are at the greatest risk for developing these cancers. In addition, men are about twice as likely to develop NMSC as women.

- **Fair-skinned people**, with light colored hair and eyes, or those more likely to burn than tan are at the greatest risk for developing these cancers. People who sunburn easily and never tan run the highest risk of skin cancer. The incidence of tumour goes up with increasing UV exposure over the USA (Scotto and Fears, 1987). Black-skinned people are less likely to get skin cancer, but they can be at risk on the soles of the feet and palms of the hands.
- **A history of sunburn** or recreational exposure to sunlight increases the risk of BCC. Risk is especially high if you had several episodes of sunburn in childhood.
- **Male gender**; men are about 2 times as likely as women to have basal cell cancers and about 3 times as likely to have squamous cell cancers of the skin (Hoban et al., 2002). This is thought to be due mainly to higher sun exposure.
- **HPV infections**; epidemiological and experimental studies have overwhelmingly confirmed human papillomaviruses (HPV) as important causal agents in NMSC carcinogenesis (Harwood and Proby, 2002; Aubin et al., 2003). HPV is found in many NMSCs, and probably contributes to the molecular mechanisms of carcinogenesis, particularly certain viral subtypes. A recent study indicates that cutaneous infections with HPV5 and HPV8 may result in an increased risk of SCC development in transplant recipients (Stockfleth et al., 2004).
- **Genetic predisposition**; the observed phenotypic diversity between individuals reflects the presence of subgroups of individuals that have a strong genetic predisposition, and may require only moderate exposure to UV to initiate tumour development, as well as others that may require intense, repeated exposures because of robust protective still unknown genetic factors.

It has been reported the prevalence of NMSC in affected families is far greater than in the normal population (Herity et al., 1989). The trait appeared to be dominantly inherited, and NMSC developed at an earlier age in subsequent generations, possibly because of a change in sun exposure habits (Czarnecki et al., 1992).

The concept of genetic susceptibility is complex. Genes are described as susceptibility genes when the mutant or variant gene is more common in the affected phenotype, or as genetic modifiers when the mutant or variant gene influences the severity of the phenotype. The selection of candidate susceptibility genes for NMSC is influenced by the various effect of UV light, this being the mayor risk factor. Thus, such genes are selected from pathways involved in DNA repair of UV-related adducts, defence against oxidative stress, immune modulation, tanning, and other related biochemical activities. Many approaches have focused on studying allele frequencies and genotype distributions of polymorphic candidate genes in case-control studies.

The human glutathione S-transferase (GST) supergene family comprises a group of genes encoding enzymes involved in the detoxification of a variety of reactive and mutagenic compounds, including the products of UV-induced oxidative damage. Polymorphisms in the Glutathione-S-transferase (GST) have been reported associated to NMSC (Heagerty et al., 1994; Lira et al., 2006), and interaction between GST allelic variants and CYP1A1 genotypes have been reported to show a higher risk of developing NMSC, especially SCC, in transplant patients, indicating that allelism and/or interactions between allelic variants at other loci may also influence the risk of NMSC (Lira et al., 2006). An association between the class II antigen HLA-DR7 and the occurrence of skin cancers has also been described. Conflicting results have been reported concerning HLA-A11: both negative and positive associations have been reported (Jensen et al., 1999).

Exposure to UV has pleiotrophic effects, both direct and indirect, in skin. It induces covalent bonds between adjacent pyrimidines generating photoproducts, such as cyclodipyrimiidine dimers (TT) and pyrimidine-pyrimidine lesions. DNA repair is important in modulating the effects of UV exposure. Thus, xeroderma pigmentosum patients defective in nucleotide excition repair systems are susceptible to multiple BCC, and they also exhibit an increased frequency of UV signature mutations in important genes, including PTCH and p53 (Grossman and Wei, 1995; D'Errico et al., 2000; Reinfenberger et al., 2005). Association of polymorphisms of PTCH gene with NMSC predisposition has also been reported (Asplund et al., 2005; Strange et al., 2004).

- Immunosupression; there is a well-defined increased risk of malignacies after solid organ transplantation (Kessler et al., 2006; Herrero et al., 2005a; Mithoefer et al., 2002;

Zafar et al., 2008) (**Tab. 1**). As increasing number of patients are transplanted and graft survival improves, the incidence of post-transplantation cancers has consequently increased (Moloney et al., 2006; Herrero et al., 2005b).

3. NMSC in transplant patients

Factors in OTRs that confer increased susceptibility to NMSC are similar to those in the general population, but the tumours behave more aggressive, occur at a younger age, are more numerous, grow more rapidly, and metastasize early in the transplant group (Sheil et al., 1985; Liddington et al., 1998; Glover et al., 1994; Martinez et al., 2003; Kovach et al., 2005) Whereas OTR share similar determinants with immunocompetent individuals, their specific tumour burden appears to be linked to the type, dosage and duration of immunosuppression. The most important predisposing factors include fair color of their skin, eyes and hair, as well as the susceptibility to sunburn. Cumulative ultraviolet radiation (especially UVB) is the primary responsible carcinogen for the induction of NMSC, as suggested by the fact that the lesions almost exclusively appear on UV-exposed skin sites, and are more numerous in patients living in sunny countries.

Age is also an important risk factor: in OTR patients, the risk ratio was reported to be around 12-fold higher in patients receiving grafts beyond age 55, compared to patients with grafts received before the age of 34 (Otley et al., 2005; Ulrich et al., 2004). While the higher prevalence of NMSC in HTR has long been thought to be due to greater immunosuppression, it seems that the older age of HTR at transplantation is the main reason (Fortina et al., 2000; Otley et al., 2005). Indeed, the dosages of immunosuppressants in HTR and KTR at the occurrence of skin cancer were recently found to be similar (Euvrard et al., 2006).

The duration of immunosuppression seems to be involved on the multiplicity of lesions, since the number of lesions per patient over the same follow-up period is significantly higher in KTR versus HTR (Euvrard et al., 2006). This could be due to the younger age at transplantation of KTR, who have a longer exposure to immunosuppression at the occurrence of the first SCC. Patients older at transplantation, even if they develop the first SCC sooner, have a shorter immunosuppression time at the first NMSC, and are more similar in this respect to the general population.

Genetic susceptibility to skin cancer may be particularly important in immunosuppressed individuals, who have the additional insult of long-term immunosuppression and increased susceptibility to HPV infections.

One possible mechanism by which UV light promotes carcinogenesis is its ability to induce prostaglandins formation, which may then function as a tumour promoter, or may enhance initiation because of their ability to act as oxidants (Vanderveen et al., 1986; Cerutti and Trump, 1999; Hruza and Pentland, 1993; Grewe et al., 1993).

4. Prostaglandin E 2

Among the various prostaglandins (PGs), PGE2 appears as a key molecule in tumour formation, because of its effects on cell proliferation, angiogenesis, immune surveillance, and apoptosis (Ali et al., 2005; Langsenlehner et al., 2006; Campa et al., 2004; Cao and Prescott, 2002; Navarro et al., 2008) (**Fig. 5**).

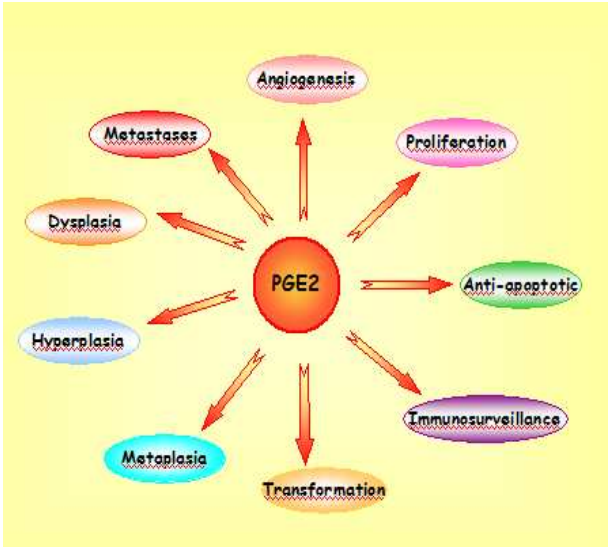


Fig. 5: Schematic representation of PGE2 possible participation in cancerogenesis (modified from Chun and Surh, 2004)

In epithelial tumours of the mammary gland, PGE2 may stimulate the formation of proangiogenic factors such as VEGF (vascular endothelial growth factor), which promotes tumour-associated angiogenesis. Solid malignancies are made up of cells that produce signals that act both in a paracrine and autocrine manner as depicted.

PGE2 can contribute to tumour development through several mechanisms including (*i*) promotion of angiogenesis, (*ii*) inhibition of apoptosis, (*iii*) increased invasiveness/motility, and (*iv*) modulation of inflammation and immune responses (Chun and Surh, 2004). The molecular mechanism responsible for PGE2-induced colorectal cancer cell migration and invasion is known to involve an epidermal growth factor receptor (EGFR)–phosphatidylinositol 3-kinase–Akt pathway. However, the mechanisms by which PGE2 modulates apoptosis are still largely unknown. One potential mechanism with regard to its regulation of programmed cell death is that PGE2 reduces the basal apoptotic rate by increasing the level of antiapoptotic proteins such as BCL-2, or other members of the

BCL gene family, such as MCL-1. In general, PGE2 suppresses immunosurveillance by down-regulating T and B cell proliferation, cytotoxic activity of natural killer cells, and cytokines such as IL-12 and tumour necrosis factor α (Wang and Dubois, 2004) (**Fig. 6**).

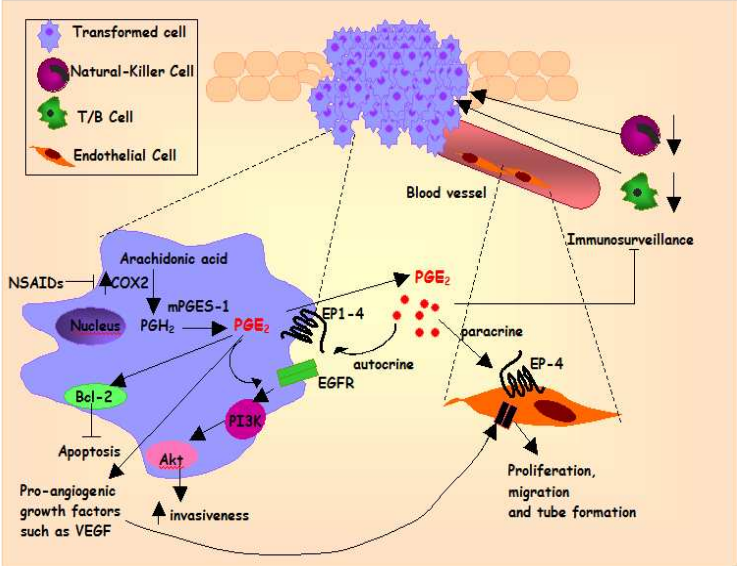


Fig. 6: Possible mechanisms of PGE2 tumorigenesis (modified from Wang and DuBois, 2004)

Depending on the tissue context, PGE2 signalling may affect growth (proliferation, terminal differentiation, and survival), angiogenesis, invasiveness, and/or immune surveillance.

The levels of extracellular-active PGE2 are regulated by metabolic clearance along an energy-dependent uptake mechanism across the cell plasma membranes through prostaglandin transporter (PGT), members of the family of organic anion transporting polypeptides (OAT), which exhibit an energy-dependent lactate/PG exchange activity. The first and rate-limiting step in the intracellular degradation of PG is catalyzed by the NAD+ dependent enzyme 15-Hydroxyprostaglandin Dehydrogenase (15-PGDH), which catalyzes the oxidation of the PG 15-hydroxy group to a 15-keto group. 15-PGDH was found to be down-regulated in human epithelial tumours and to show tumour suppressor activity in epithelial cancers (Müller-Decker and Fürstenberger, 2007).

PGE2 is produced via three sequential enzymatic reactions: release of arachidonic acid (AA) from membrane glycerophospholipids by phospholipase A2 (PLA2), conversion of AA to the unstable intermediate prostanoid PGH2 by cyclooxygenase (COX), and isomerization of PGH2 to PGE2 by prostaglandin E synthase (PGES) (**Fig. 7** and **Fig. 8**).

As autocrine and/or paracrine signalling molecules, PGE2 is released from cells and it interacts with distinct G protein-coupled receptors (GPCR) characterized by seven-transmembrane domains. PGE2 may bind to the four GPCR isoforms EP1, EP2, EP3, and EP4.

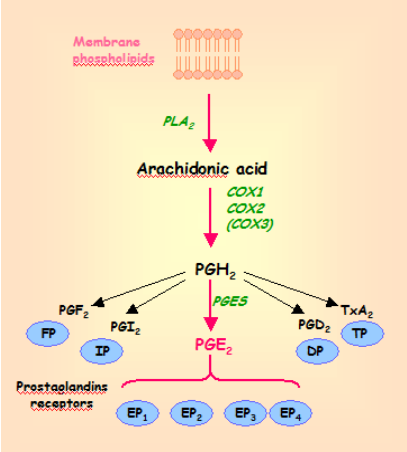


Fig. 7: schematic representation of the prostaglandins biosynthetic pathway from membrane phospholipids (modified from Chell et al., 2006)

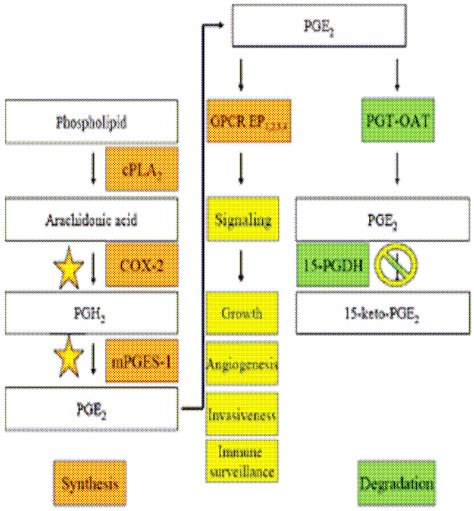


Fig. 8: PGE_2 signalling in cancer. The stars correspond to COX2, and mPGES-1 over-expression in a wide range of premalignant and malignant epithelial tumours (Müller-Decker and Fürstenberger, 2007).

5. Cyclooxygenase 2 (Cox-2)

An over-expression of Cox-2, which results in excessive prostaglandin production, has been observed in human skin actinic keratoses (premalignant UV-induced skin lesions), SCCs, BCCs, in human epidermal keratinocytes after UVB injury, in squamous cell skin cancer, in human epidermal cancer cell lines (Buckman et al., 1998; Subbaramaiah and Dannenberg, 2003; An et al., 2002; Higashi et al., 2000; Hull, 2005; Tripp, et al., 2003), and in early stages of carcinogenesis in a wide variety of tissues. A mouse model of UV-induced skin carcinogenesis utilizes SKH-1 hairless mice, which initially develop benign papillomas and then SCCs after chronic UVB exposure. In this model, Cox-2 has also been shown to be over-expressed early on in UV-induced hyperplastic skin as well as in papillomas and SCCs (An et al., 2002; Pentland et al., 1999). Moreover, COX-2 has been shown to be transiently induced in human and mouse skin after a single acute

exposure to UV radiation (An et al., 2002; Tripp et al., 2003; Akunda et al., 2007; Fischer et al., 2007).

Coincident with Cox-2 up-regulation, primarily in the basal layer, is an increase in PGE2 production and an increase in keratinocyte proliferation and apoptosis (Akunda et al., 2007; Fischer 2007; Trifan and Hla, 2003; Cao and Prescott, 2002).

To demonstrate that UV induction of Cox-2/PGE2 expression is important for UV-induced skin responses and carcinogenesis, various nonsteroidal anti-inflammatory drugs (NSAIDs), which are inhibitors of COXs, have been used in the SKH-1 and other mouse models. Both indomethacin, a general Cox inhibitor, and celeCoxib, a Cox-2-selective inhibitor, when fed to mice in the diet, were found to significantly inhibit UV-induced tumorigenesis in terms of number of tumours/mouse and tumour size (Pentland et al., 1999; Fischer et al., 1999). Similarly, topical treatment of mice with celeCoxib immediately following each UV irradiation, reduced the number of tumours/mouse and tumour size (Wilgus et al., 2003). Papillomas that did develop on mice treated with UV plus topical celeCoxib had reduced levels of PGE2and reduced the number of proliferating cells and p53-positive cells compared with papillomas from mice not treated with celeCoxib (Wilgus et al., 2003). Even when celeCoxib treatment (dietary or topical) was initiated after tumours had developed and UV exposures were stopped, celeCoxib was able to inhibit new tumour formation, although there was little or no regression of established tumours (Pentland et al., 1999; Wilgus et al., 2003; Fischer et al., 2003). Moreover, it has been shown that regular users of NSAIDs have a reduced risk of developing actinic keratoses and cutaneous SCCs (Butler et al., 2005).

Currently, three Cox isoenzymes are known: Cox-1, Cox-2 and Cox-3. Cox-3 is a splice variant of Cox-1 which retains intron 1 and has a frameshift mutation.

In terms of their molecular biology, Cox-1 and Cox-2 are of similar molecular weight (approximately 70 and 72 kDa respectively), and share approximately 60-65% amino-acid identity with each other; Cox-1 orthologs (without the signal peptide) share approximately 70-95% amino-acid identity across vertebrate species, and Cox-2 orthologs share 70-90%. The most significant difference between the isoenzymes, which allows for selective inhibition, is the substitution of isoleucine at position 523 in Cox-1 with valine in Cox-2. The relatively smaller Val_{523} residue in Cox-2 allows access to a hydrophobic side-pocket in the enzyme (which Ile_{523} sterically hinders). Drug molecules, such as DuP-697 and the Coxibs derived from it, bind to this alternative site and are considered to be selective inhibitors of Cox-2 (Mifflin and Powel, 2001, http://www.medpubinc.com/newsletters/RPL.pdf) (**Fig. 9**).

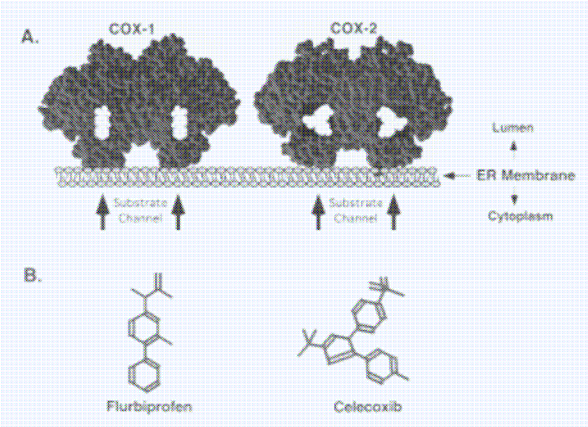


Fig. 9. A: Diagrammatic representation of the of Cox-1 and Cox-2 in the ER membrane. In the central portion of each monomer is shown a cutout section demonstrating NSAID binding to the active site. In the case of COX-1, the active site is narrower allowing access only to NSAIDs with smaller side chains. Cox-2 specific NSAIDs contain larger side chains not accommodated by the Cox-1 pocket. **B:** Diagrammatic representation of the molecular structure of flurbiprofen, a nonselective NSAID, and celecoxib, a Cox-2 specific NSAID (Mifflin and Powel, 2001, http://www.medpubinc.com/newsletters/RPL.pdf)

Cox-1 is normally constitutively expressed in most tissues and its expression usually does not vary greatly in the adult animal (Smith et al., 2000). The PG products of COX-1 are involved in normal physiologic functions, such as maintenance of the gastric mucosa and regulation of renal blood flow (**Fig. 10**).

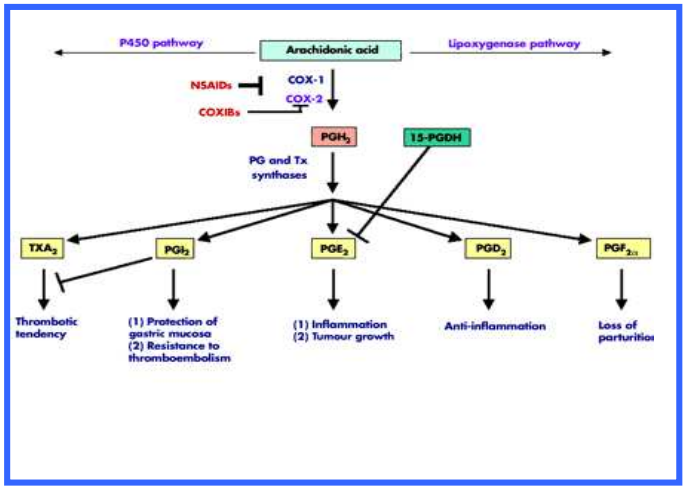


Fig. 10: Overview of prostaglandin (PG) synthesis and main functions (Wang and DuBois, 2006).

Cox-2 expression, on the other hand, is generally undetectable in most unperturbed adult tissues, but can be highly induced by a variety of agents, including proinflammatory agents and mitogens (Taketo, 1998; Smith et al., 2000; Hull, 2005).

To date, Cox-2 has been determined to contribute to tumorigenesis and to the malignant phenotype of tumour cells via the inhibition of apoptosis, increased angiogenesis and invasiveness, and modulation of inflammation and immunosuppression (Dempke et al., 2001; Wang and Dubois 2006; Sahin et al., 2009).

5.1 PTGS2 gene

The PTGS2 gene (also named COX2) maps to chromosome 1(1q25.2-25.3), contains ten exons (**Fig. 11**) and codes for a protein of 604 aa: the inducible cyclooxygenase Cox-2 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PTGS2). PTGS2 is an immediate-early response gene whose expression is highly induced in response to hormones, pro-

inflammatory cytokines, growth factors, oncogenes, carcinogens and tumour promoters (Smith et al., 2000; Hull, 2005).

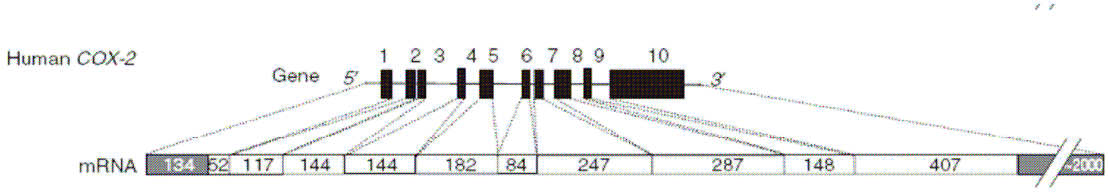


Fig. 11: Schematic representation of PTGS2 gene (also named COX2) and corresponding mRNA (Chandrasekharan and Simmons, 2004).

PTGS2 expression is regulated at both transcriptional and post transcriptional level, and the deregulation of PTGS2 expression observed in carcinogenesis can in part be due to functional changes affecting these regulatory regions of the gene.

The promoter region of the PTGS2 gene contains several key cis-acting regulatory elements, including a canonical TATA box, and various response elements for nuclear factor kB (NK-kB), PEA3, AP2, nuclear factor interleukin-6 (NF-IL6), Sp1, adenosine 3', 5'-cyclic-monophosphate-response element (CRE), c-MYB, transforming growth factor β (TGFβ), and others (**Fig. 12**). Depending on the stimulus and the cell type, these transcription factors can play a decisive role in the regulation of COX-2 transcription (Shao et al. 2000; Dixon, 2003a; Chun and Surh, 2004; Yang and Bleich 2004).

Current studies have demonstrated that post-transcriptional regulation plays a central role in the regulatory mechanisms of PTGS2 expression (Harper and Tyson-Capper, 2008). The PTGS2 3′UTR is larger than average, encompassing ~2.5 kb, and has many interesting features. It has several polyadenylation signals, only two of which are commonly used, resulting in mRNAs of ~2.8 or ~4.6 kb. The proximal polyadenylation signal has a non-consensus CPSF (cleavage polyadenylation specificity factor) binding site (AUUAAA), followed by putative upstream enhancer elements (USEs). It is likely that regulation occurs in part here, resulting in two mRNAs with different RNA metabolism. The significance of the different tissue distributions of these isoforms has not yet been addressed, and tissue specificity might play a role in PTGS2 polyadenylation site choice (Cok and Morrison, 2001).

The 3′UTR also has 22 repeats of adelylate and uridylate-rich (AU-rich) elements (AREs), composed of the sequence 5'-AUUUA-3. This AU-rich region is highly conserved in both sequence and location among human, mouse, rat, chicken, pig, cow and sheep PTGS2 mRNA transcript, implying that the function of the ARE have been evolutionary conserved. This element, which is present within the 3'UTRs of many proto-oncogene and

cytokine mRNAs, confers post-transcriptional control of expression. There are many different polypeptides that specifically interact with AREs from rapidly degraded mRNAs. These regulatory trans-acting factors include several cytoplasmic mRNA-binding proteins proposed to be involved with the destabilization, stabilization, or mRNA processing and nucleocytoplasmic transport (Cok and Morrison 2001; Dixon 2003a; Dixon et al., 2003b). Regulation of gene expression at a post-transcriptional level is also mediated by microRNAs, short single-stranded non-coding RNAs, which affect stability and translation of their target mRNAs (Jing et al., 2005).

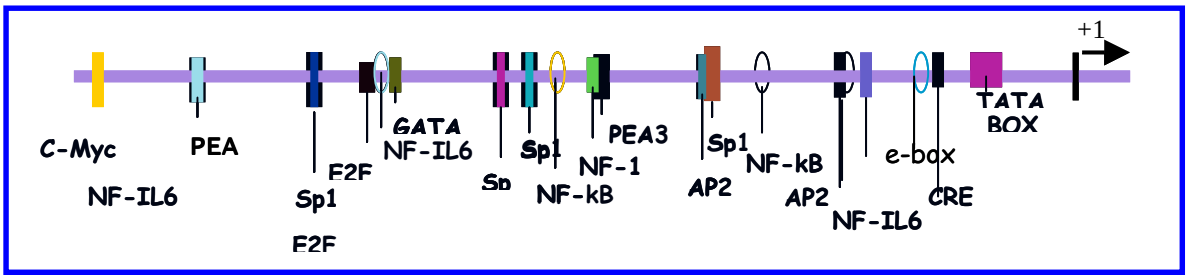


Fig. 12: Schematic representation of the proximal region of human PTGS2 promoter. The TATA box is shown as a box and the transcription binding sites are depicted as bars and circles. The transcription start site (+1) is indicated by an arrow.

5.1.1 PTGS2 polymorphisms –765G>C and 8473T>C

One common polymorphisms in the promoter region of the PTGS2 gene (-765G>C) (**Fig. 13**) has been reported to alter promoter activity (Papafili et al., 2002, Zhang et al., 2005). Polymorphism -765G>C (rs 20417) is located at a putative Sp1 binding site and it has been shown that allele -765C have significantly lower promoter activity compared with -765G in human lung fibroblasts cells. This polymorphism has been associated to myocardial infarction and stroke, to asthma risk, and to the risk of esophageal cancer (Cipollone et al., 2004; Papafili et al., 2002; Zhang et al., 2005).

One common polymorphism in the 3'UTR region (8473T>C) (**Fig. 13**) has been described and reported associated to an increased risk for non-small cell lung cancer, colorectal adenoma, and breast cancer (Langsenlehner et al., 2006; Campa et al., 2004; Cox et al., 2004). In contrast, three other studies described a protective effect of the same genetic variant against lung cancer (Hu 2005; Sorensen et al., 2005; Park et al., 2006). The functionality of polymorphism is currently unknown and the reason for this discrepancy could be due to ethnic differences between studies. The 8473T>C (rs 5275)

polymorphism could be in linkage disequilibrium with other causal genetic variants, and this linkage disequilibrium would likely be different across different ethnic populations.

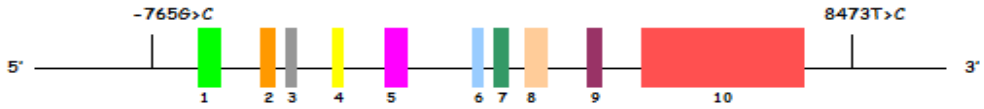


Fig. 13: Schematic representation of PTGS2 gene showing the relative position of polymorphisms -765G>C and 8473T>C.

6. Prostaglandin E synthase (PGES)

Despite a rapidly expanding body of information on the structures, expression and regulatory functions of various eicosanoid biosynthetic enzymes during the last decade, little has been learned about the molecular identity of PGES until very recently.

Three forms of PGES have been identified to date, cytosolic PGES, microsomal PGES-1 and microsomal PGES-2 (Tanioka et al., 2000; Jakobsson et al., 1999; Tanikawa et al., 2002).

Cytosolic PGES (cPGES) is constitutively expressed in many tissues and preferentially coupled with COX1 in the maintenance of tissue homeostasis (Tanioka et al., 2000). Microsomal PGES-1 (mPGES-1) is induced by proinflammatory stimuli (Jakobsson et al., 1999), and displays a marked preference for being functionally coupled with COX2 (Mancini et al., 2001), contributing to a variety of physiological and pathologic conditions, such as fever, inflammation, and reproduction (Murakami et al., 2000; Murakami et al., 2002; Yamagata et al., 2001). Transcript for mPGES-2 is more abundantly distributed in the brain, heart, skeletal muscle, kidney and liver than in other tissues (Tanikawa et al., 2002). In contrast to the marked inducibility of mPGES-1, mPGES-2 is constitutively expressed in various cells and tissues and is not increased appreciably during tissue inflammation or damage (Murakami et al., 2003). When transfected in several cell lines, mPGES-2 is coupled with both COX-1 and COX-2, leading to PGE2 production (Murakami et al., 2003) (**Fig. 14**). Since the mature mPGES-2 exists as an N-terminally truncated form that is dispersed in the cytoplasm, mPGES-2 can function as a cytosolic enzyme rather than a membrane-bound enzyme.

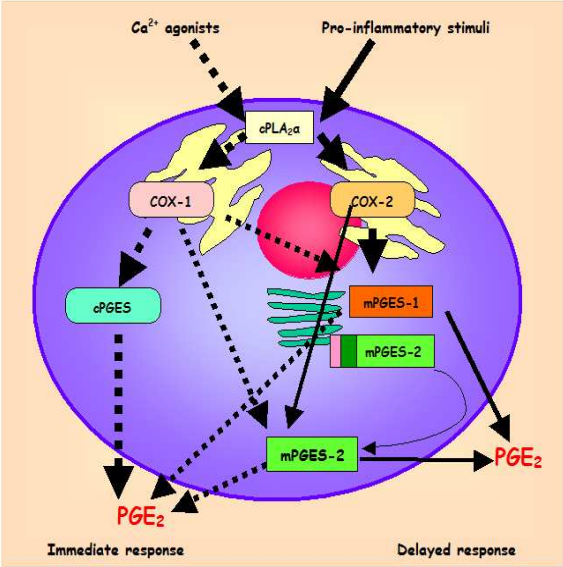


Fig. 14: Coupling between two COX isoforms and three PGES enzymes (modified from Murakami and Kudo, 2006).

The mPGES-1 enzyme is a member of the MAPEG (membrane associated proteins involved in eicosanoid and glutathione metabolism) superfamily, together with FLAP (five-lipoxygenase-activating protein) and leukotriene C4 synthase, proteins which are also involved in arachidonic acid metabolism. The primary structures of proteins from several species show a high degree of sequence homology (**Fig. 15**). mPGES-1 also shows significant homology with other MAPEG superfamily proteins, including MGST-1, MGST-2, MGST-3, 5-lipoxygenase-activating protein (FLAP) and leukotriene C4 synthase (LTCS), with the highest homology being found with MGST-1 (~40%). mPGES-1 requires glutathione (GSH) as an essential cofactor for activity (Jakobsson et al., 1999; Murakami et al., 2000). Two-dimensional crystals of purified mPGES-1 demonstrate that the enzyme structure is trimeric (Thorén et al., 2003).

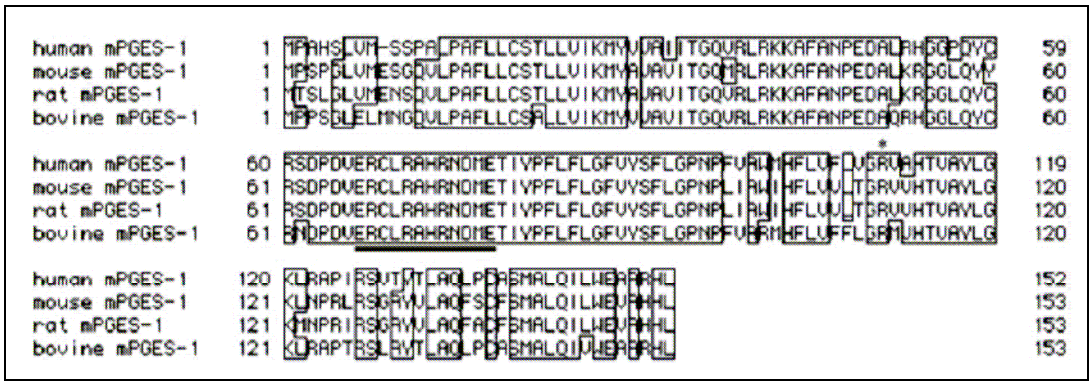


Fig. 15: Primary amino acid sequences of mPGES-1. Residues conserved in all four species are boxed. The Arg residue essential for catalytic activity is shown by asterisk, and the putative MK-886-binding motif is underlined (Murakami and Kudo, 2006).

6.1 mPGES-1 gene

The human mPGES-1 gene maps to chromosome 9q34.3, is 14.7 kb long and contains three exons (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PTGES&search=ptges) (**Fig. 16**). Although this gene is probably co-stimulated with COX2, the promoter region of mPGES-1 lacks a TATA box and contains different transcriptional elements not present in the COX2 promoter (**Fig. 17**). In the mPGES-1 proximal promoter region there are two GC boxes, two tandem Barbie boxes, and an Aryl Hydrocarbon Response element (AHR), consistent with other members of the MAPEG and Glutathione-*S*-transferase families (Forsberg et al., 2000). The 3' region of the mPGES-1 gene lacks the AUUUA mRNA instability sequences that are found in the COX-2 gene. More detailed analysis of ~1800 bp upstream of the human and mouse mPGES-1 gene reveals binding sites for C/EBPa and b, AP- 1, and two tandem GC-boxes as well as two progesterone receptor- and three glucocorticoid receptor-responsive elements (Naraba et al., 2002). Of these sites, the proximal GC boxes are most critical for the inducible promoter activity. Expression of mPGES-1 is in part regulated by the mitogen-activated protein kinase pathways (Han et al., 2002), where the kinases may switch to the proximal GC boxes in the mPGES-1 promoter, leading to inducible mPGES-1 transcription (Naraba et al., 2002). Additionally, mPGES-1 expression is ablated in lipopolysaccharide (LPS)-stimulated macrophages from NF-IL-6-deficient mice (Uematsu et al., 2002), indicating the participation of this transcription factor in the induction of mPGES-1. Both the Sp1 and Sp3 proteins are important for the basal expression of mPGES-1 (Ekström et al., 2003).

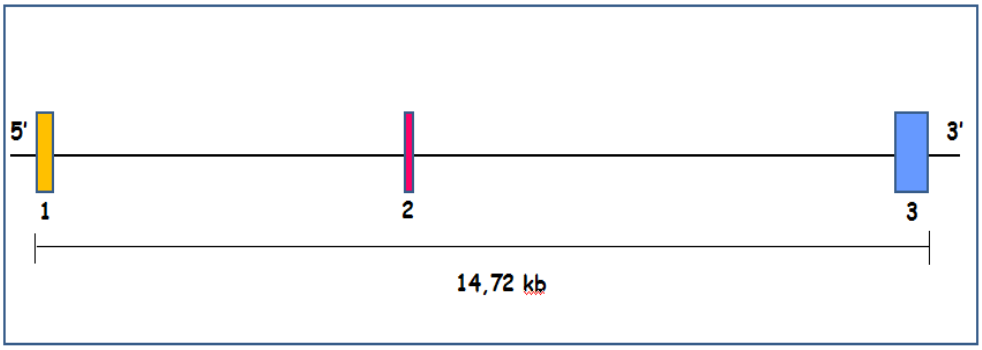


Fig. 16: Schematic representation of mPGES-1 gene

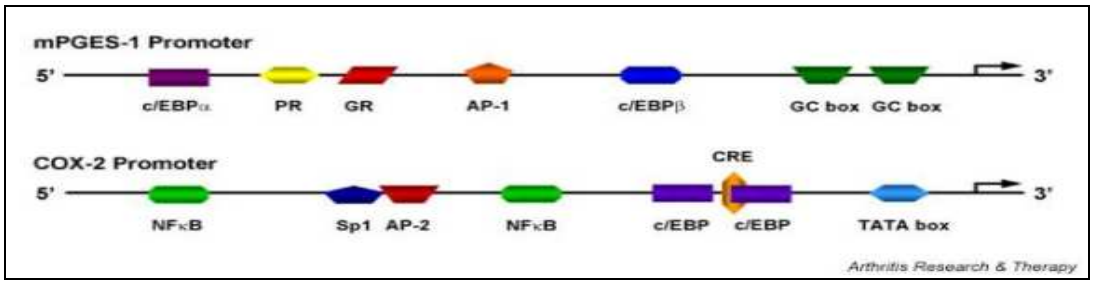


Fig. 17: Regulatory elements of the human microsomal prostaglandin E synthase-1 (mPGES-1) and cyclooxygenase-2 promoters. AP-1 or -2, activator protein-1 or -2; c/EBP, CCAAT/enhancer-binding protein; GR, glucocorticoid receptor; NFκB, nuclear factor κB; PR, progesterone receptor; Sp1, specificity protein-1 (Sampey et al., 2005).

Although the steady-state expression of mPGES-1 in normal rat tissues is very low, administration of lipopolysaccharide (LPS) leads to a dramatic increase in mPGES-1 expression in various tissues (Murakami et al., 2000; Mancini et al., 2001; Yamagata et al., 2001). Stimulation of various cultured cells with pro-inflammatory stimuli leads to a marked elevation of mPGES-1 expression, often with concomitant induction of COX-2 (Jakobsson et al., 1999; Murakami et al., 2000; Mancini et al., 2001). Transfection of mPGES-1 in combination with COX-2, but not with COX-1, into Human Embryonic Kidney 293 cells (HEK293 cells) leads to cellular transformation, which is manifested by aggressive cell growth, piling up and aberrant morphology, with a concomitant increase in PGE2 (Murakami et al., 2000). Mutations affecting expression or activity of mPGES-1 could result in alteration in the response to specifics stimuli, leading to a differential inflammatory reaction, and could be associated to many diseases where inflammation plays an important role.

An over-expression of mPGES-1 has been reported in cancer cells of various human and mouse tissues, such as non-small cell lung cancer (NSCLC), head and neck squamous cell carcinoma, squamous cell carcinoma of the penis, and papilloma lesions of the skin (Yoshimatsu et al., 2001; Cohen et al., 2003; Golijanin et al., 2004; Neumann et al., 2007).

6.1.1 mPGES-1 polymorphisms

Screening of the coding sequence of the gene identified two non-synonymous polymorphisms, only one likely to be functionally relevant, present among African-Americans and not among the Caucasian populations. (Bigler et al., 2007) Polymorphisms in the coding sequences of the gene are rare and and largely of low allele frequency, suggesting little tolerance of genetic variations that could affect normal prostaglandin functions.

Few polymorphisms in the non coding sequence of the gene have been reported in relation with hypertension and breast cancer with no positive results (Iwai et al., 2004; Abraham et al., 2009).

To our knowledge, the possible association of mPGES-1 genetic variants on the risk of NMSC has never been investigated.

7. EP receptors

PGE2 acts via four cell-surface seven-transmembrane G-protein-coupled receptors known as E prostanoid (EP) receptors, designated EP1 through EP4. Prostanoid receptors are generally expressed in cell surface plasma membranes, although recent data suggests some EP receptor subtypes to be also associated with the nuclear membrane.

In particular, the perinuclear localization of native EP1 receptor protein has been reported in porcine cerebral microvascular endothelial cells, as well as cloned EP1 in Swiss 3T3 fibroblasts (Bhattacharya et al., 1998). Other receptors (particularly EP3 and EP4) have since also been suggested to be functionally expressed in the nuclear membranes of neonatal porcine brain and adult rat liver cells (Bhattacharya et al., 1999). The presence of nuclear EP receptors therefore implies that prostanoids may be capable of acting intracellularly in a novel form of autocrine signalling with a potentially greater capacity to influence nuclear events, as well as their established ability to signal in a conventionally autocrine and paracrine way.

Although little information exists about the expression and function of the EP receptors in the skin, the differential expression of these receptors during the development of UVB induced SCC in both experimental animals and humans indicates that these receptors are important in mediating the effects of prostaglandins in UVB-induced SCC.

The possible role of the EP receptors in the photocarcinogenesis process is proposed in **Fig. 18.** Induction of COX-2 expression by UV irradiation results in increased production of prostaglandin E2 (PGE2). PGE2 binds to and activates four G protein-coupled receptors, of which EP1, EP2 and/or EP4 appear to be important for the effect of PGE2 in UV-induced skin damage and carcinogenesis.

Signalling through the EP receptors results in the induction of various genes, including oncogenes, cytokines and growth factors, which then lead to inflammation with infiltration and activation of inflammatory cells in the dermis, induction of angiogenesis, vasodilation

and vascular permeability, leading to edema, increased keratinocyte proliferation and a reduction in apoptosis. All of these downstream effects of Cox-2 over expression and PGE2 signalling promote the development of UV-induced skin carcinogenesis (Rundhaugand Fischer, 2008).

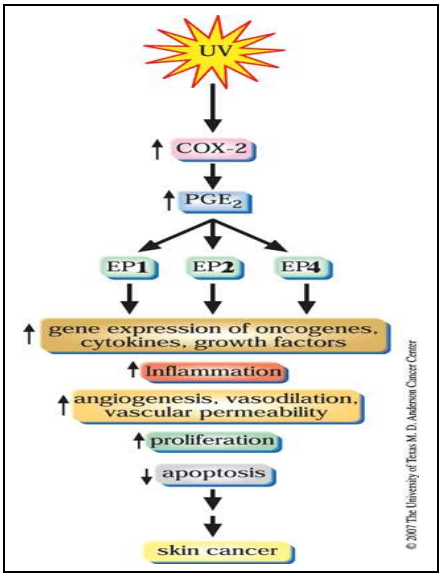


Fig 18: possible role of the EP receptors in the photocarcinogenesis process (Rundhaug and Fischer, 2008)

In particular, an increase in the level of EP1 receptor expression was observed in UVB-induced benign papillomas and SCC in murine skin (Lee et al., 2005) and immunohistochemical analysis has demonstrated that EP1 is expressed by differentiated keratinocytes in murine epidermis(Tober et al., 2006).

Moreover, the inhibition of the EP1 receptor through topical application of a specific EP1 antagonist, ONO-8713, successfully decreased the infiltration of neutrophils into the skin in response to acute UVB exposure, and significantly reduced UVB-induced tumour development (Tober et al., 2006).

7.1 PTGER1 gene

The EP1 receptor is coded by the PTGER1 gene which maps to chromosome 19p13.1. This gene is 2897bp long and contains three exons (http://www.genecards.org/cgi-bin/carddisp.pl?gene=Ptger1) (**Fig. 19**).

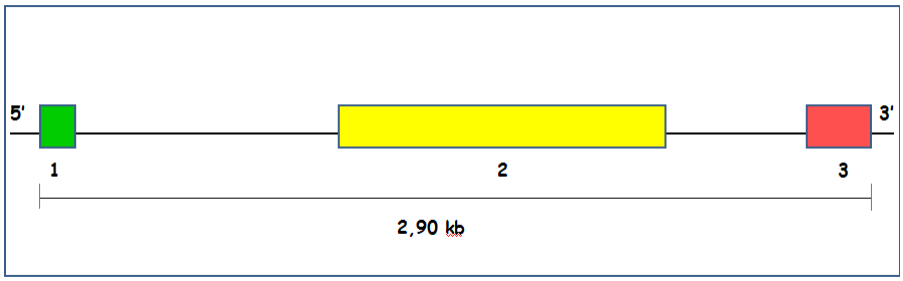


Fig. 19: Schematic representation of PTGER1 gene

The presumed promoter region of the mouse EP1 receptor gene does not contain a TATA box or consensus sites for mammalian CCAAT-binding proteins, but does contain several GC-box-like motifs. These features are characteristic for many TATA-less promoters. In the proximal region of the transcription start site, there is also one consensus AP-1 site (TGAGTCA) and three AP-2 sites (GSSWGSCC) (Batshake et al., 1995) (**Fig. 20**).

```
-1443                                                    CTTGGGGTTCAGACCTGGTTCCCCACTCACCCAGGCCTCACATA
-1399  TGCTCCTCTTGTGTGAGAGCCCCCGTGTGTGGCAGCAAGGCCTGTTGCCCTGTTACCCTAGTTGTACTCAGGGGACAGGCATCCATGGCACAGTGCTTCA
-1299  CAGAGATGTAAACCTGCCAAGCTTGTTTTGACAGTCCATTTCATCCTAGGATTTAAGGAGATCAAGGAAAGGGTGGGGTGGGACCCTGGCCAGACTTGGG
-1199  GAGAGGGGAGGCAATTGGAACAAGGGGAACCAGGCCAGAGGTCTCTCTCCAGCAACCATAGGAATGTTTGGGGTAAGCCCAGCAGAGATGCTGCAGGAAG
-1099  GCTGAGTGGTACTGGTTCCCTGGTACCAGGCAGGAGATGGGTATCCTGGATATAGGACAGAAGCCCTGCAGATGGCTACTTGCTATCCCTGATCACAAGC
 -999  CCATTTTCAGTACTGATAGTAGAACCCAGGGCTTCATACATGCTAAGGATGTGCTCTGTCACTGAACTGTAGCCCAGGTCCTATACTGGGGTCTCGCATG
 -899  TCTGCTGGGAGCAGCTGGGACCATGTGTAGTGGGTGGTTGGTAGCCAGGTTGAACCAGAGCTGTGATTATTGTGGGTAAGCCTTGGGTGCAGTGAGTTTC
 -799  AGGGAGCTGTGTTCAGTGTGTGCATGGGTCTCTGGTGTATTCTTTCTTTTTTTTGGGGGGGGGGGGATTCAGGATAAACAGGCCTTAGAGATGTGATTAT
 -699  GTGTCTCAGGACAAGTTCTCGGGGTCCATGGGCATTGGTTTAAGGGAGACTACATCAGGAGGTGTGCTCTGGGGCTGGGAACAGACTTACAGTGACAGTG
 -599  AGATTTACACCCAGTGCTGAGGTAAGCATCAGTTTCATATGGGGGAGGGGGGATAGATGTTACAGTCAGCAATGGTGAGTGCTATCCAGTGTTTACTCCC
 -499  CATTATGACTAAGCTGACAGTGGCTGCATGTGTGCCCCTTTTCAAGACAGAAGGTGAGACATAGCTATGGATACAGGGTGCCCTATGAATTGCCTACCTC
 -399  CGCATAAGGCAGGATACAGATGATAGCCTGGCTCCCCTGAGTCATTTAGCCACCTCTCTTCACCTAGGATGACTCCCTGTTGCCCTGGTCTAGGCTGCCC
 -299  ACCTCTGCCCAACTGTGACAACCATAAAGGCAAGGGTAGGAGGCATTGCTAGCTCCCATCACACCCAGGCAACTCCCAACCCCTCAAGTCCAATGAGGAC
 -199  AACACTCCTCCACACACACACTCACTCTCCCGCCCCCCACGGGAACTCATAGCCCTTCCAGCCTGCCCCAGGCGATGCTCACATTTTCCAGTGTTTAGAT
  -99  TTTATCCACTTTATTAATGAGGCGGGCAAGAGGCCCGGGTGAGGGTGTGGGGGTTTGCTCCTGCCCCGCTGAGAGGAGGGGGGTCACAGAGACCAAACCA
    1  ATTCAGAGAACCTCAGTCCTTGGCGGGAAGGAGCTGGGCCCAGTTGCCCTGACTGTTGCCAGCAGTTTTGCCCACTCAAGGCTCCCAGCCCCAGAGAGCA
```

Fig. 20: Nucleotide and deduced amino acid sequence of the 5' of the mouse EP, receptor gene. The arrow indicates the transcription initiation site. Nucleotides are numbered relative to the transcription initiation site (+ 1) The potential binding sites for transcription factors **AP-1** and **AP-2** are single and double underlined, respectively (from Batshake et al., 1995).

Cloning of the human EP1 receptor has revealed a protein of 402 amino acids with a predicted molecular weight of approximately 42 kDa (Watabe et al., 1993). Like the other EP receptors, it is a seven-transmembrane G-protein-coupled receptor (**Fig. 21**).

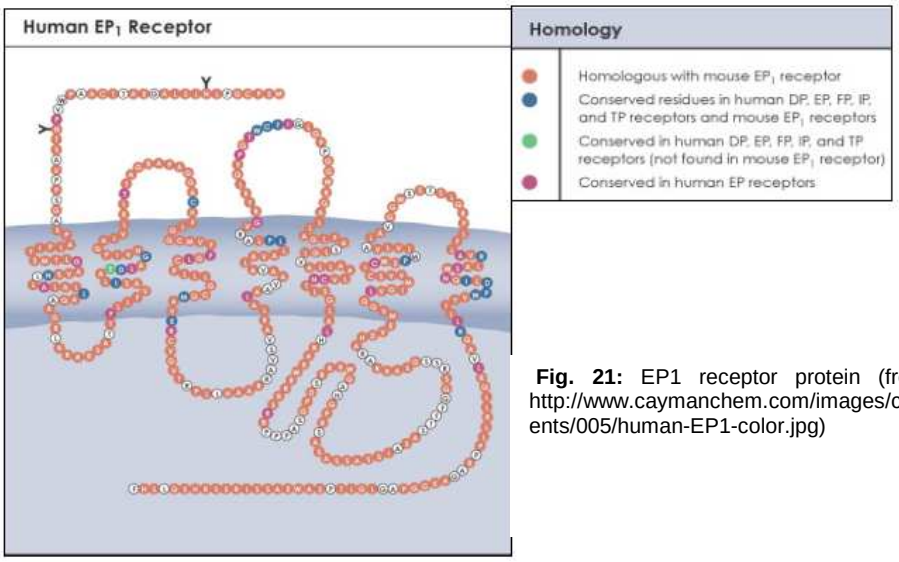


Fig. 21: EP1 receptor protein (from http://www.caymanchem.com/images/currents/005/human-EP1-color.jpg)

7.1.1 PTGER1 polymorphisms

Screening of the coding sequence of the human gene identified two non-synonymous polymorphisms, only one likely to be functionally relevant, present among African-Americans and not among the Caucasian populations (Bigler et al., 2007).

Association studies regarding polymorphisms in the PTGER1 gene have only been reported in relation to aspirin intolerant asthma (AIA) in the Korean population, where one common polymorphism in the downstream region of the gene (rs2241363) appeared to be associated to a lower risk of AIA (Park et al., 2010).

Although different studies suggest the possible involvement of the EP1 receptor in skin tumorigenesis (Lee et al., 2005; Tober et al., 2006), to our knowledge, the possible association of PTGER1 genetic variants with the risk of NMSC has not yet been investigated thus far.

8. Molecular mechanisms by which UVB light can induce PTGS2, mPGES-1 and PTGER1 expression

Several studies have shown a link between prostaglandin production and the initiation and progression of skin carcinogenesis (Fischer et al., 1999; Wilgus et al., 2003; Chun et al., 2007). UVB activates a number of signalling pathways leading to inflammatory gene

expression, a process referred to as 'the UV response' (Heck et al., 2004). Initially characterized by activation of the immediate early genes, c-fos and c-jun and various transcription factors including NFκB (Heck et al., 2004), more recent studies have demonstrated that PI3K/Akt and MAP kinase signalling are also key to the response (Bode and Dong, 2003), and this response appears to be differentiation-dependent. In fact, while the ERK1/2, JNK, p38 MAP kinases and Akt were activated by UVB in undifferentiated cells, in differentiated cells, only JNK and p38 kinase were activated. Inhibition of p38 MAP kinase has been shown to suppress UVB-induced inflammation in mouse skin including expression of proinflammatory cytokines and Cox-2 production (Hildesheim et al., 2004; Kim et al., 2005). Black et al., 2008 observed that UVB-induced PTGS2 expression is regulated via p38 kinase, as well as JNK and Akt kinase, and that UVB-induced expression of mPGES-1 was suppressed by JNK and PI3K/Akt inhibition. UVB light stimulates the release of arachidonic acid from keratinocyte cell membranes, and increases the expression of PTGS2 and various prostaglandin synthases and EP1 receptor (**Fig. 22**). This indicates that UVB not only upregulates the capacity of keratinocytes to generate PGE2, but also their responsiveness to this prostaglandin.

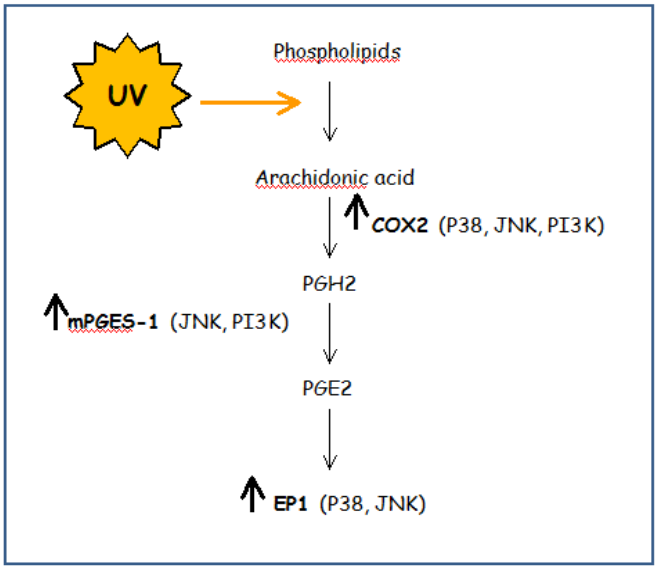


Fig. 22: Summary of the effects of UVB on the PGE2 biosynthetic pathway (Black et al., 2008).

The deregulation of the expression of PTGS2, mPGES-1 and PTGER1 can in part be due to functional changes of the regulatory elements of gene expression.

This study was designed to investigate if polymorphisms of PTGS2, mPGES-1 and PTGER1 (encoding the EP1-receptor) can constitute risk factors for the development of NMSC in OTRs, and to determine if these polymorphisms can be useful genetic markers of susceptibility allowing to early highlight individuals at greater risk of NMSC, after transplantation.

In order to achieve this goal, polymorphisms were genotyped to define their association with the phenotype.

MATERIALS AND METHODS

1. Subjects

Transplanted patients (kidney, heart and liver) undergoing immunosuppressive therapy with a functioning graft and a minimum of one year of follow-up since transplantation, and affected by at least one histologically proven NMSC within 10 years from transplantation, were considered cases. Controls were OTRs free from skin cancer for a minimum of 10 years after transplantation. For each patient, basic demographic data, date of transplantation and of the visit, long term immunosuppressive therapy, type of skin cancers, skin type, eye and hair colour were recorded. Long term maintenance immunosuppression consisted in a combination of one or two immunosuppressive drugs (azathioprine, cyclosporine, mycophenolate mofetil and tacrolimus) with oral methylprednisolone, according to current protocols (Halloran, 2004). A total of 286 solid organ transplant recipients, including 144 cases and 142 controls, were selected for the study (**Tab. 2**).

Skin tumours were classified by pathological criteria.

Tab. 2: Study group characteristics

	Cases	**Controls**	**Total**
Sex			
M/F (total patients)	125/19	117/25	242/44
Type of transplantation	N°	N°	N°
Kidney	107	120	227
Heart	31	21	52
Liver	6	1	7
Mean ± SD age at transplantation (years)	45.33 ± 13.34	45.01 ± 13.04	46.2 ±12.99
(Range)	(17.84-72.87)	(12.28-63.59)	(12.28-72.87)
Type of cancers (cases)	N°		
Basal Cell Carcinoma (BCC)	63		
Squamous Cell Carcinoma (SCC)	68		
Bowen	4		
Keratoacanthoma	3		
Merkel cell carcinome	3		
Others	3		

2. Molecular methods

DNA was extracted from peripheral blood leukocytes by standard salting out method (Miller et al., 1988).

2.1 Genetic analysis

2.1.1 PTGS2

To determine –765G>C (rs20417) genotype, Amplification Refractory Mutation System-PCR (ARMS-PCR) was performed. **Tab. 4** describes the primers used, designed according to Ensembl, ID ENSG00000073756. As a polymerase chain reaction (PCR) control, we co-amplified a SPINK 1 gene fragment of 317 bp.

Genotyping of polymorphism 8473 T>C (rs5275) was performed by Restriction Generating PCR (RG-PCR) (Gasparini et al., 1992), using a Forward modified primer that creates a Bcl I restriction site when allele 8473C is present (**Tab. 4**). The digestion products were analysed by 3% agarose gel electrophoresis.

2.1.2 mPGES-1

Search for new polymorphisms in the mPGES-1 promoter was performed by amplifying a 1166 bp region, spanning position −1193 upstream the starting site of transcription to position -27, in seven partially overlapping fragments. Primer sequences were designed based on the mPGES-1 genomic sequence available from Ensembl, ID ENSG00000148344 (**Tab. 3**).

Amplified genomic fragments were screened for mutations using heteroduplex analysis by electrophoresis in Mutation Detection Enhancement gels (MDE Gel Solution, Biospa Società Prodotti Antibiotici S.p.a., Milan, Italy), with 15% (w/v) urea, at 250 V for 16–24 h. Amplified fragments with altered migration pattern were directly sequenced at the BMR Genomics of Padua.

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (PCR-RFLP) analysis were performed to genotype polymorphism –664T>A (rs7873087), using primers and endonuclease as detailed in **Tab. 4**. Amplification products of four individuals homozygous -664AA (minor allele) and 25 random individuals with genotype –664TA were directly sequenced at the BMR Genomics of Padua.

Genotyping of polymorphism –439T>C (rs7872802) was performed by RG-PCR, using a Forward modified primer that creates a Rsa I restriction site when allele –439C is present. **Tab. 4** describes the primers used, designed according to Ensembl, ID

ENSG00000148344. The digestion products were analysed by 3% acylamide gel electrophoresis.

Tab. 3: Primers used for the amplification of segments in the proximal 5' region of the mPGES-1 gene

	Primers	Amplified fragment
Fragment 1	pF: 5'-gctggaattacaggtgtgag-3' pR: 5'-tgagacccatttcaggcttc-3'	276 bp
Fragment 2	pF: 5'-ctcaggaagtcagtggagcc-3' pR: 5'-ggagagacgctaacctgctg-3'	282 bp
Fragment 3	pF: 5'-gagagtttgtttcgtcccct-3' pR: 5'-gtgactatgtggcaaaaggg-3'	301 bp
Fragment 4	pF: 5'-gaagggcccctctgtatact-3' pR: 5'-cacactcagcctggacaatg-3'	231 bp
Fragment 5	pF: 5'-catggatgtctttaggggtg-3' pR: 5'-ctgccttctagggaatgatg-3'	225 bp
Fragment 6	pF: 5'-ataatcaccacgtgcagagg-3' pR: 5'-gagaaagacaagcagttgcc-3'	224 bp
Fragment 7	pF: 5'-ccacatgctcccacactgta-3' pR: 5'-tgtgatcagctcgacagagg-3'	286 bp

2.1.3 PTGER1

Polymorphisms of the PTGER1 gene (coding for the EP1-receptor) were selected from the SNPper database. Polymorphisms –1760C>A (rs3810254), -1728A>G (rs3810255), –1113C>T (rs2241359) in the PTGER1 gene were genotyped by PCR-RFLP.

Tab. 4 describes primers and restriction enzymes used. Primers were designed according to Ensembl, ID ENSG00000160951.

Tab. 4: Primers and endonucleases used for genotyping polymorphisms of the PTGS2, mPGES-1 and PTGER1 genes. Undelined characters are the modified bases.

Gene	Polymorphism	Primers	Restriction enzyme	(bp)
PTGS2	**-765G>C**	PF1:5′aggagaatttacctttcccc3′ PF2:5′aggagaatttacctttcccg3′ pR:5′ggctgtatatctgctctatatg3' SpinkF:5'tttgagttcatcttacaggtgag3' SpinkR:5'cagggtcatatggcagatggcagcaa3'	—	206
	8473T>C	pF: 5'-gtttgaaattttaaagtacttttg**a**t-3' pR: 5'-atgtctagatttcaaattattgtttcattgc-3'	BclI	147
mPGES-1	**–664T>A**	pF: 5'-gagagtttgtttcgtcccct-3' pR: 5'-gtgactatgtggcaaaaggg-3'	FblI	301
	–439T>C	pF: 5'-ag**a**ctgagtgt**a**ggggcg**t**a-3' pR: 5'-ctgccttctagggaatgatg-3'	RsaI	140
PTGER1	**–1760C>A**	pF: 5'- tcttag**t**gtgtgaggctg**c**g-3' pR: 5'-gccacagcgagtcatatcct-3'	HhaI	198
	-1728A>G	pF: 5'-tgccagtgtgaccctacttc-3' pR: 5'-gccacagcgagtcatatcct-3'	HinfI	180
	–1113C>T	pF: 5'-ctgc**a**act**a**caggcccccc**c**g-3' pR: 5'-cactgcctgggatgtggggt-3'	SacII	188

2.2 Functional analysis

The promoter activity of the proximal 5' flanking region of the PTGER1 gene was investigated in vitro by Luciferase reporter gene constructs transiently transfected in cultured cells of Mycoplasma free HeLa cells and human keratinocyte cell line HaCat.

2.2.1 Reporter gene constructs

Two fragments of 1452 bp (from –2461 bp to –1012 bp) and 212 bp (from –1221 bp to –1012 bp), respectively, were amplified using forward and reverse primers that introduce a restriction site for endonuclease Xho I and Hind III, respectively (**Tab. 5**).

Amplified products and pGL3 basic Luciferase vector (LUC, Promega) were digested with endonucleases Xho I and Hind III (**Fig. 23**), and purified from agarose gel after electrophoresis, using Qiagen purification kit. Quantification of the purified products was determined using NanoDrop®ND-1000. Ligation of amplified products to pGL3 basic Luciferase vector was performed overnight using the T4 ligase (Promega). JM109 competent cells (Stratagene) were transformed with the ligation products. Sequences of positive identified clones were confirmed by sequencing.

Tab. 5: Primers used for the amplification of the two fragments of the PTGER1 gene. Primers are modified (underlined characters) to introduce a restriction site for endonuclease Xho I and Hind III

Gene	Fragment	Primers
PTGER1	1452 bp	pF:5'*gcgctcgag*agggcaattgcaggtgtaact3' pR: 5'*ctgaagctt*tctccggcaagccgcttc3'
	212 bp	pF:5' *gcgctcgag*caactccagggagctca 3' pR: 5'*ctgaagctt*tctccggcaagccgcttc3'

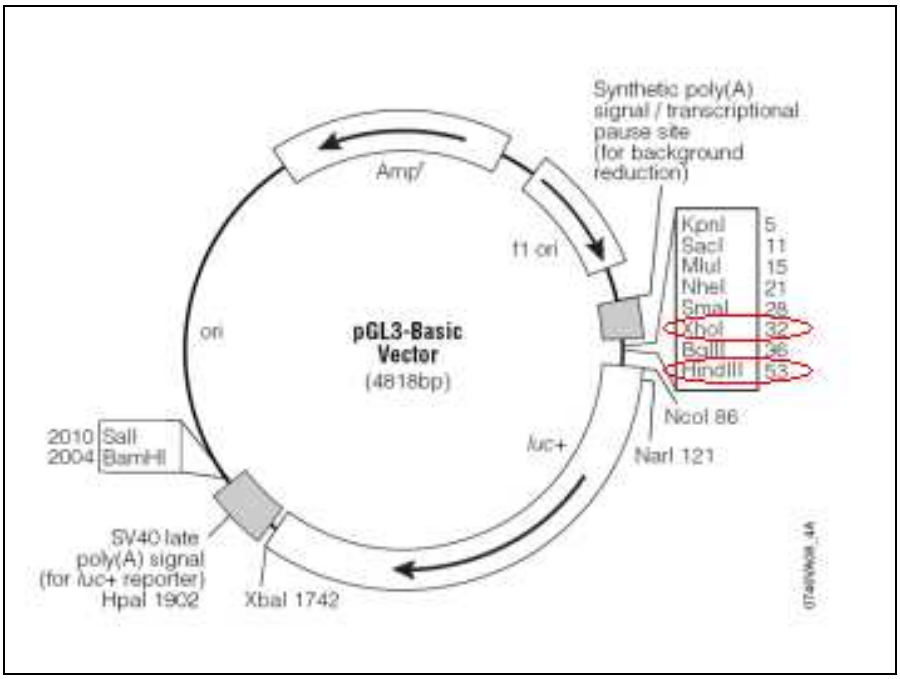


Fig. 23: pGL3 basic vector red circles indicate the used endonucleases

2.2.2 Cell culture

Cells were cultured in DMEM (Dulbecco Modified Eagle's Medium) with 10% deactivated FCS (fetal calf serum), Glutamine 1%, 100U/ml Penicillin-100ug/ml Streptomycin at 37℃ with 5% CO_2.

2.2.3 Transfections

Cells were seeded in 24-wellplate at a density of $6x10^4$ cell/well. When cultures achieved 50-70% confluence, the cells were co-transfected with 1µg of LUC construct, together with 40ng of the plasmid pRL-null-vector, which contains the gene for Renilla reniformis luciferase (pRL), and serves as control for transfection efficiency. Negative control was obtained using pGL3-basic vector without any insert. Transfections were performed with SuperFect Transfection Reagent (Qiagen), according to the manufacturer's instructions. Three hours after transfection, the medium was changed, and Hacat cells were cultured 5 or 20h in the presence or absence of LPS (LPS 300ng/mL; LPS 100ng/mL; no LPS). Promoter functionality in HeLa cells was performed without LPS stimulation. After incubation, Firefly and Renilla lucifereses activity were measured using "Dual-Luciferase Reporter Assay System" (Promega), according to the manufacturer's instructions. LUC and pRL activity were measured sequentially using a $20/20^n$ luminometer (Turner

Biosystem). Promoter activity was normalized by dividing LUC activity by pRL activity. Experiments were carried out in triplicate.

3. Statistical analysis

Genotypes for all polymorphisms were measured and the Epi Info software package (version 2003) was used for assessing association using 2x3 and 2x2 contingency tables. Chi-square test, with Yates' correction or Fisher's exact test was used for association. Odds ratio (OR) and the OR 95% confidence interval were also calculated. Single locus analysis was performed in the total group of NMSC. We also tested the hypothesis of association to SCC and BCC, since the etiology of SCC and BCC might be very different and by stratifying individuals by age < or > 50 years at transplantation, since age is an important risk factor for NMSC (Navarro et al., 2008). The correction of the p value threshold, by Bonferroni multiple test adjustment, is difficult to establish, since tests in the study group are not independent. We have accepted for single gene the threshold of 0.05/5=0.01.

Interaction among risk alleles was evaluated, by logistic analysis models, for polymorphisms –765G>C (rs20417) and -1728A>G (rs3810255) to assess the contribution to NMSC.

The significance of functional results was determined by Student's T-test and p values were evaluated. Values are expressed in mean value ± standard deviation.

RESULTS

1. PTGS2

1.1 Genotype analysis

Observed genotype distributions of the polymorphisms –765G>C and 8473T>C are summarized in **Tab. 6**. A frequency of 0.19 and 0.35 was observed for polymorphisms –765G>C and 8473T>C, respectively, in the overall population sample. Single locus analysis of polymorphisms showed no differences in genotype distributions between cases and controls. Stratification of the population by age at transplant showed that, in BCC who underwent transplantation before 50 years of age, allele –765C was never present in homozygosity, and only one heterozygote case was found. Individuals with GG genotype appear to have a major risk of BCC development [(p=0.015; OR=10.31 (1.36-209.74)] (**Tab. 6**).

Tab. 6: genotype distribution of PTGS2 polymorphisms in cases and controls as total NMSC and stratified by BCC, SCC and age at transplant

	-765G>C					8473T>C				
	GG	**GC**	**CC**	**Total**	**p value**	**TT**	**TC**	**CC**	**Total**	**p value**
Controls	95	41	6	142		64	59	19	142	
NMSC	97	38	9	144	0.7	57	66	17	140	0.64
BCC	46	14	3	63	0.61	28	26	8	62	0.99
SCC	43	20	5	68	0.61	24	34	8	66	0.39
					<50 years					
Controls	76	31	4	111		51	47	13	111	
NMSC	33	9	2	44	0.62	19	21	4	44	0.79
BCC	22	1	0	23	0.02[a]	13	8	2	23	0.65
SCC	9	8	2	19	0.14	5	12	2	19	0.22
					>50 years					
Controls	19	10	2	31		13	12	6	31	
NMSC	64	29	7	100	0.94	38	45	13	96	0.64
BCC	24	13	3	40	0.98	15	18	6	39	0.81
SCC	34	12	3	49	0.74	19	22	6	47	0.66

[a] GG vs GC+CC p=0.015; OR=10.13 (1.36-209.74)

2. mPGES-1

2.1 Search for polymorphisms

Heteroduplex analysis of amplified fragments covering a region of 1166 bp of the proximal 5´ region of the gene was performed in 30 SCC, 30 BCC and 30 control individuals.

Direct sequencing of fragments presenting altered migration pattern demonstrated three variants −664T>A (rs7873087), –663A>T (rs7859349) and –439T>C (rs7872802).

2.2 Genotype and haplotype analyses

NCBI database reported equal frequencies for the polymorphisms -663A>T and -664T>A, suggesting linkage disequilibrium between these two variants. This hypothesis was supported by sequence analysis of 4 homozyotes, and 25 random heterozygote individuals. Consequently, only polymorphism -664T>A was genotyped.

Observed genotype distribution (**Tab. 7**) of polymorphism –439T>C indicated complete linkage disequilibrium also between this SNP and -664T>A (overall frequency=0.14). Single locus analysis showed no differences in genotype distributions between cases and controls, neither when analysing NMSC as a total group of cases, nor when stratified by type of tumour or by age at transplant (**Tab. 7**). Haplotype analysis of the overall population showed no differences in haplotype distributions between cases and controls (**Tab. 8**).

Tab. 7: genotype distribution of mPGES-1 polymorphisms in cases and controls as total NMSC and stratified by BCC, SCC and age at transplant

mPGES-1 genotype	Controls	NMSC	BCC	SCC
TT_{-664}-AA_{-663}-TT_{-439}	82	82	35	39
TA_{-664}-AT_{-663}-TC_{-439}	27	27	11	14
AA_{-664}-TT_{-663}-CC_{-439}	3	1	0	1
Total	112	110	46	54
p value		0.61	0.53	0.92
	<50 years			
TT_{-664}-AA_{-663}-TT_{-439}	64	19	8	10
TA_{-664}-AT_{-663}-TC_{-439}	21	9	4	4
AA_{-664}-TT_{-663}-CC_{-439}	3	1	0	1
Total	88	29	12	15
p value		0.74	0.66	0.79
	>50 years			
TT_{-664}-AA_{-663}-TT_{-439}	18	63	27	29
TA_{-664}-AT_{-663}-TC_{-439}	6	18	7	10
AA_{-664}-TT_{-663}-CC_{-439}	0	0	0	0
Total	24	81	34	39
p value		0.78	0.69	0.95

Tab. 8: distribution of mPGES-1haplotypes in cases and controls as total NMSC and stratified by BCC, SCC and age at transplant

mPGES-1 haplotype	Controls	NMSC	BCC	SCC
T_{-664}-A_{-663}-T_{-439}	191	191	81	92
A_{-664}-T_{-663}-C_{-439}	33	29	11	16
p value		0.73	0.64	0.88
	<50 years			
T_{-664}-A_{-663}-T_{-439}	149	47	20	24
A_{-664}-T_{-663}-C_{-439}	27	11	4	6
p value		0.66	0.77	0.59
	>50 years			
T_{-664}-A_{-663}-T_{-439}	42	144	61	68
A_{-664}-T_{-663}-C_{-439}	6	18	7	10
p value		0.99	0.94	0.82

3. PTGER1

3.1 Genotype and haplotype analyses

The three most frequent polymorphisms in the proximal 5' region of the gene were selected from the SNPper database: –1760C>A (rs3810254), -1728G>A (rs3810255), and –1113C>T (rs2241359). The frequency reported in NCBI database for these common variants is 0.14 (CEU low coverage panel).

Genotype distribution for the three polymorphisms is reported in **Tab. 9**. A frequency of 0.14 was observed for the three polymorphisms, showing complete linkage disequilibrium for all the three variants. Haplotype analysis of the overall population showed no differences in haplotype distributions between cases and controls (**Tab. 10**).

Genotype distribution showed an association trend (p=0.049) to SCC in individuals who underwent transplantation before 50 years of age. Individuals carrying the three minor alleles were more represented in cases than in controls.

Tab. 9: genotype distribution of PTGER1 polymorphisms in cases and controls as total NMSC and stratified by BCC, SCC and age at transplant

PTGER1 genotype	Controls	NMSC	BCC	SCC
CC$_{-1760}$-GG$_{-1728}$-CC$_{-1113}$	86	76	29	39
CA$_{-1760}$-GA$_{-1728}$-CT$_{-1113}$	27	32	16	14
AA$_{-1760}$-AA$_{-1728}$-TT$_{-1113}$	0	2	1	1
Total	113	110	46	54
p value		0.22	0.09	0.32
		<50 years		
CC$_{-1760}$-GG$_{-1728}$-CC$_{-1113}$	69	19	7	11
CA$_{-1760}$-GA$_{-1728}$-CT$_{-1113}$	20	9	5	3
AA$_{-1760}$-AA$_{-1728}$-TT$_{-1113}$	0	1	0	1
Total	89	29	12	15
p value		0.13	0.15	0.049
		>50 years		
CC$_{-1760}$-GG$_{-1728}$-CC$_{-1113}$	17	57	22	28
CA$_{-1760}$-GA$_{-1728}$-CT$_{-1113}$	7	23	11	11
AA$_{-1760}$-AA$_{-1728}$-TT$_{-1113}$	0	1	1	0
Total	24	81	34	39
p value		0.86	0.66	0.93

Tab. 10: distribution of PTGER1haplotypes in cases and controls as total NMSC and stratified by BCC, SCC and age at transplant

PTGER1 haplotype	Controls	NMSC	BCC	SCC
C$_{-1760}$-G$_{-1728}$-C$_{-1113}$	199	184	74	92
A$_{-1760}$-A$_{-1728}$-T$_{-1113}$	27	36	18	16
p value		0.23	0.11[a]	0.58
		<50 years		
C$_{-1760}$-G$_{-1728}$-C$_{-1113}$	158	47	19	25
A$_{-1760}$-A$_{-1728}$-T$_{-1113}$	20	11	5	5
p value		0.19[b]	0.19[c]	0.37
		>50 years		
C$_{-1760}$-G$_{-1728}$-C$_{-1113}$	41	137	55	67
A$_{-1760}$-A$_{-1728}$-T$_{-1113}$	7	25	13	11
p value		0.93	0.69	0.85

[a] OR=0.56 (0.28-1.13); [b] OR=0.54 (0.23-1.31); [c] OR=0.48 (0.15-1.66)

3.2 Functional analysis

Functional analysis of the proximal 5' of the gene where the three polymorphisms are located was performed by two constructs containing 1452 bp and 212 bp, respectively. Both constructs showed promoter activity when transiently transfected in HeLa cells (**Fig. 24**). When HaCat cells were transiently transfected in the presence/absence of LPS, the two constructs didn't show promoter activity (**Fig. 25-26**). LPS stimulation with 300ng/mL or 100ng/mL did not change these results.

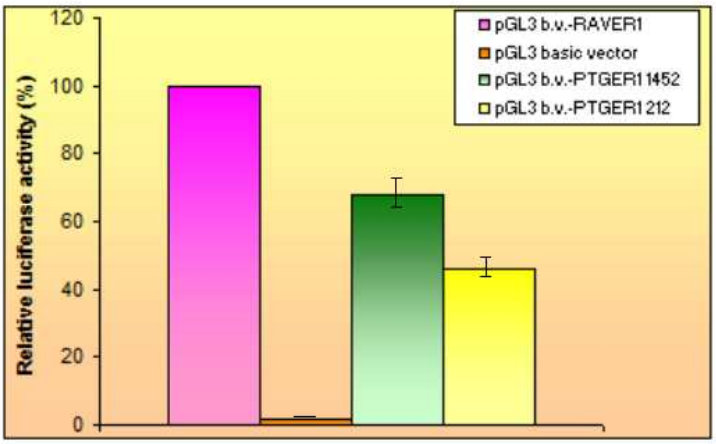


Fig. 24: functional analysis in HeLa cells of two regions of the proximal 5'region of PTGER1. The pGL3 basic vector-RAVER1 promoter construct has been used as positive control.

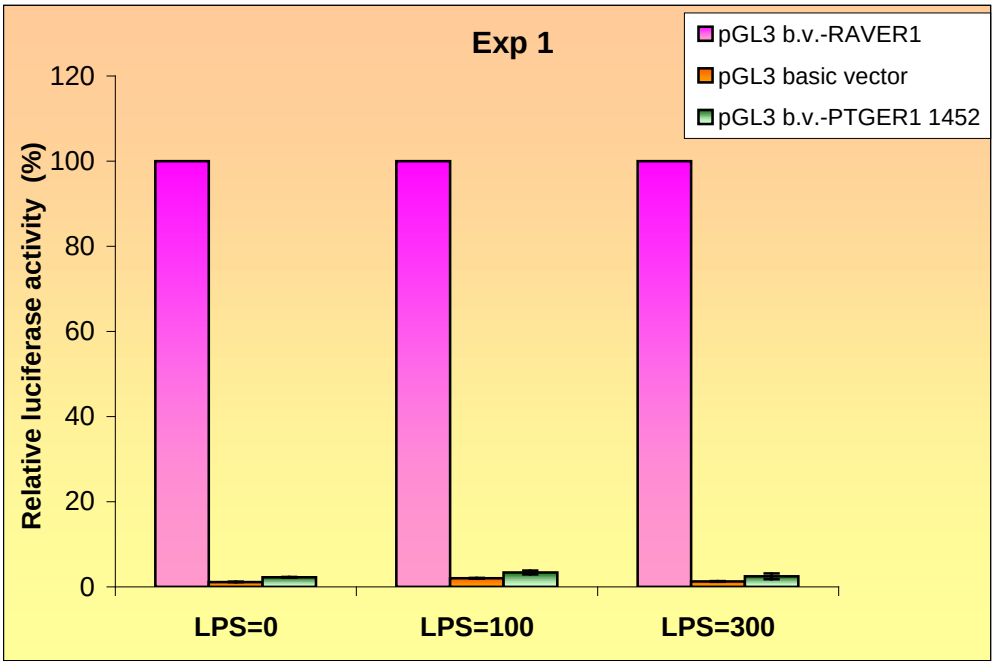


Fig. 25: functional analysis of the 1452bp region of 5'proximal of PTGER1 in HaCat cells in the presence/absence of LPS for 20h.

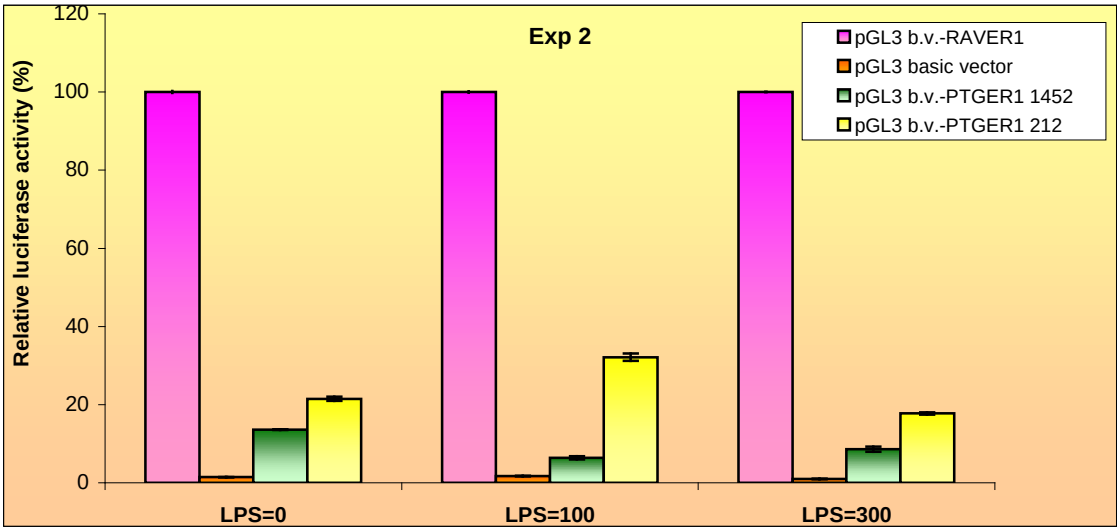


Fig. 26: functional analysis of the two regions of the proximal 5'region of PTGER1 in HaCat cells in the presence/absence of LPS for 5h.

DISCUSSION

The involvement of PGE2 in tumorigenesis, by promoting cell survival, cell growth, migration, invasion and angiogenesis, is supported by multiple lines of evidence. The synergic action of cyclooxygenase (Cox), and prostaglandin E synthase (PGES) is required for the synthesis of PGE2, which exerts its effects by interacting with four receptors (EP1-4).

Until recently, COX expression was believed to be the major determinant of PGE2 levels, but recent data indicate that multiple levels of control exist for the regulation of PGE2 production and signalling. In addition to regulation via COX enzymes, PGE2 synthesis can be regulated via expression of specific prostaglandin E synthases (PGES), which act on the COX product, prostaglandin H2, to produce PGE2 (Chell et al., 2006 ; Park et al., 2006b). The cellular response to PGE2 can also be regulated via the differential expression of the four PGE2 receptors (EP1–4), which activate distinct downstream signalling responses (Chell et al., 2006; Hull et al., 2004). An understanding of these levels of regulation (and response to) of PGE2 synthesis may reveal novel strategies for prevention and/or treatment of cancer. Genetic association studies can also provide insights into pathogenic and protective mechanisms, and identify new molecules and pathways which may become targets for pharmacological intervention. Moreover, genetic association studies could contribute to the identification of clinical and genetic markers of susceptibility that could allow early detection of individuals at highest risk to diseases, in order to focus on them with preventive efforts, subjecting them to a closer follow up, a more effective health education, and possibly preventive drug therapy.

The inducible form of COX (Cox-2) and microsomal-PGES-1 (mPGES-1) are over-expressed in cancer of various tissues, and PGE2-receptors (EP1-4) appear differentially expressed during skin cancer development induced by UVB, with an over-expression of EP1 (Lee et al., 2005). Early response genes are often regulated at both, transcriptional and post transcriptional levels, and their deregulation can in part be due to functional changes affecting the regulatory regions of the genes. Transcription process is complex, and requires the co-ordinated action of multiple regulatory proteins through complex protein–DNA and protein–protein interactions (Orphanides and Reinberg, 2002). Variation in the DNA sequence may potentially alter the affinities of existing protein–DNA

interactions or, indeed, recruit new proteins to bind to the DNA, altering the specificity and kinetics of the transcriptional process (Tournamille et al., 1995; Knight et al., 1999). Alteration in gene expression due to genetic variants in PTGS2 (coding for Cox-2), mPGES-1, or PTGER1 (coding for EP1) may thus play a role in the predisposition of NMSC in OTRs.

The present study was initially focused on polymorphisms -765G>C *(*rs20417) and 8473T>C (rs5275) in the PTGS2 gene, since these common polymorphisms, located in the transcriptional and post-transcriptional regulatory regions, respectively, have been reported to be associated with various human malignancies (Zhang et al., 2005; Langsenlehner et al., 2006; Campa et al., 2004; Cox et al., 2004; Hu 2005; Sorensen et al., 2005; Park et al., 2006; Lira et al., 2007).

In a previous study, OTRs and control cases were genotyped for polymorphisms –765G>C (rs20417) and 8473T>C (rs5275) in the PTGS2 gene and association of polymorphism –765G>C with a subgroup of NMSC was reported (Lira et al., 2007).

In the present study, we extended the published report to a larger number of patients, and the selection of patients was done introducing the cut-off of 10 years after transplantation. In agreement with the former work, allele –765G in the PTGS2 gene seems to represent a risk factor in the development of BCC tumours in individuals undergoing transplantation before 50 years of age [p=0,015, OR 9.59 (1.36-192.66)]. We speculate that the protective effect of the counterpart, allele -765C, is probably abolished in elderly individuals, where the additive effects of risk factors accumulation and diminished DNA repair capacity exert stronger effects. The correction of the p value threshold, by Bonferroni multiple test adjustment, is difficult to establish, since tests in the study group are not independent. We have accepted for single gene the threshold of 0.05/5=0.01. In SCC, the effect of variant –765C is not evident, indicating that possibly environmental and genetic risk factors may play different roles in the outcome of these two types of lesions.

The common polymorphism 8473T>C (rs5275) in the PTGS2 3'UTR region of the transcript has been described associated with several cancers in different ethnic populations with controversial results (Liu et al., 2010). Although different studies have reported the association of this variant to different malignancies, no information concerning its functionality is available. Previous work done in our laboratory, for the first time, analysed, in human keratinocyte cell line HaCaT the possible role of 8473T>C on PTGS2 gene expression. Transient transfection of construct vectors containing 8473T or C alleles did not showed any differences in gene expression (unpublished data). Since

regulation of PTGS2 gene expression depends on the type of cell and stimulus, it is possible that this variant may have no, or little, effect on post transcriptional regulation of PTGS2 expression in transformed human keratinocytes.

One possible explanation for the discrepancy between association studies concerning 8473T>C could be found in differences in linkage disequilibrium between these two SNPs across different ethnic groups, being polymorphism -765G>C the possible real causal genetic variant.

It is noteworthy that, according to the HAPMAP data for the CEU population, the markers -765G>C and 8473T>C in the studied sample are in linkage disequilibrium (LD) (D'= 0.898 and r^2 =0.351). In this study we did not observed any association of 8473T>C with the predisposition to NMSC. This result is not surprising since that, to identify an association of 8473T>C due to its linkage disequilibrium to -765G>C, a much greater number of individuals would be necessary.

Despite a rapidly expanding body of information on expression and regulatory functions of PTGS2, little has been learned about mPGES-1 until very recently. Mutational analysis and association studies of the mPGES-1 gene have been reported in very few phenotypes. Polymorphisms in the non coding sequence of the gene have been reported in relation with hypertension and breast cancer, with no positive results (Iwai et al., 2004; Abraham et al., 2009). These data indicate that mPGES-1, such as other genes of the prostaglandin biosynthetic pathway, is highly conserved, with little tolerance for genetic variations. The prostaglandin synthesis pathway is vital for the normal functioning of the human body, and probably deleterious mutations have been evolutionarily selected against, and are rare in this gene (Bigler et al., 2007).

The present case-control study describes the analysis of the mPGES-1 genetic variability in the proximal promoter region, in relation to NMSC after transplantation. Over-expression of mPGES-1 has been observed in various cancer tissues (Yoshimatsu et al., 2001; Cohen et al., 2003; Golijanin et al., 2004; Neumann et al., 2007). In particular, mPGES-1 expression was reported to be upregulated by UVB in undifferentiated and differentiated keratinocytes (Black et al., 2008).

To our knowledge, this is the first study where the proximal promoter region of the gene has been systematically screened for polymorphisms, and where the possible association of mPGES-1 genetic variants with the risk of NMSC after organ transplantation have been analysed. Three polymorphisms, reported in NCBI databases as –664T>A (rs7873087), –663A>T (rs7859349) and –439T>C (rs7872802), were identified.

All three polymorphisms are located within conserved regions of the gene, in four catarrhini primates (Homo sapiens, Pan troglodytes, Pongo pygmaeus and Macaca mulatta, Ensembl) sequences. Polymorphisms within conserved regions are likely to be of greater phenotypic significance than those within more divergent regions. As assessed by bioinformatic TESS Job, the presence of variants -664A and -663T may abolish a nuclear factor erythroid derived 2 (NF-E2) binding site, and may create a globin transcription factor-1 (GATA-1) binding site. NF-E2 is involved in the hemoglobin production (http://www.ncbi.nlm.nih.gov/omim/601490) and GATA-1, one of the six members of the GATA gene family, has a crucial role in normal human hematopoiesis (http://www.ncbi.nlm.nih.gov/omim/305371). Extensive survey of human tissue samples found that NF-E2 expression is not limited to erythropoietic organs. Expression in the colon and testis suggested that NF-E2 may participate in the regulation of genes other than globin (Chan et al., 1993). Mutations in GATA-1 have been found to have important clinical significance, and are directly linked to deregulated formation of certain blood cell lineages (Ciovacco et al., 2008). Although the potential effect of –664T>A, –663A>T and –439T>C polymorphisms on gene expression, no association with NMSC predisposition was observed. A minor effect of the mPGES-1 gene on the NMSC susceptibility cannot be ruled out, mainly due to the sample size analyzed.
Although we evaluated the possible impact of the three sequence variants using *in silico* methods, functional studies are needed to define the influence of these polymorphisms on gene expression.

The events downstream the enhanced PGE2 secretion that drive the carcinogenic process remain thus far largely undefined. PGE2-receptors (EP1-4) are differentially expressed during skin cancer development induced by UVB (Lee et al., 2005). An over-expression of EP1, as a result of enhanced PGE2 levels, has been reported in both murine and human UVB-induced NMSC (Lee et al., 2005).
Screening of the coding sequence of the human PTGER1 gene has identified two non-synonymous polymorphisms, only one likely to be functionally relevant, present among African-Americans and not in the Caucasian populations (Bigler et al., 2007). The promoter region of the human PTGER1 gene has not yet been characterized. The presumed promoter region in the mouse gene showed one consensus AP-1 site and three AP-2 sites (Batshake et al., 1995), located in conserved regions of the gene, aligning Homo Sapiens and Mus Musculus sequences. Since the promoter activity of this region has not been tested in humans, we performed functional analysis to investigate if the region containing the analyzed polymorphisms has any transcriptional regulatory

function. Although a weak promoter activity was observed in HeLa cells, we were unable to demonstrate promoter activity of this region in HaCat cells even after LPS stimulation.
Association studies regarding polymorphisms in the PTGER1 gene have only been reported in relation to aspirin intolerant asthma (AIA) in the Korean population, where one common polymorphism in the downstream region of the gene (rs2241363) appeared to be associated to a lower risk of AIA (Park et al., 2010).
Although the possible involvement of the EP1 receptor in skin tumorigenesis has been suggested (Lee et al., 2005; Tober et al., 2006), the possible association of PTGER1 genetic variants with the risk of NMSC has not yet been investigated thus far.
We focused our attention on polymorphisms –1760C>A (rs3810254), -1728G>A (rs3810255), and –1113C>T (rs2241359), in the 5' flanking region of the PTGER1 gene, since these variants have a reasonable frequency in the Caucasian population (0.14 in NCBI database), and/or are located within conserved regions of the gene. In particular the –1113C>T polymorphism is located in a putative binding site for a transcription factor (AP-2 in mouse and AP-2 alpha in human, TESS Job program http://www.bx.psu.edu/old/courses/bx-fall05/tess.html). Analysis of the three polymorphisms showed complete LD, and the haplotype containing the three minor variants was more represented in SCC individuals when compared to matched controls who underwent transplantation before 50 years of age, showing a trend of association in this subtype of patients.
Additional association studies in an extended cohort of individuals or in other study populations would be necessary to verify the consistence of these results.

Complex diseases, such as NMSC, can be due to the additive effects of different variants in several genes that, taken singly, would show only small effects on disease onset and pathogenesis. Thus, we have evaluated, in our cohort of patients, a possible synergistic effect of the polymorphisms analysed. Logistic analysis did not show evidence of any joint effect when possible risk alleles of PTGS2 and PTGER1 genes were considered.
A big limitation of this work is the small sample size, which would have enabled us to identify only major at risk alleles, with a considerable possibility to fall in error type 2 (false negative). Although our results do not support the hypothesis that polymorphisms in the mPGES-1, and PTGER-1 genes could represent important risk factors for NMSC development, these data could be useful for further meta-analysis studies, where also small cohort of patients are considered. The difficulties to include these results in meta-analysis studies are mainly due to the inability to get them published, since 'negative'

(non-statistically significant) results are more likely to remain unpublished than studies with 'positive' (formally statistically significant) results. This is one of the biggest limitations of meta-analyses, since they often introduce publication bias.

In conclusion, allele –765C in the PTGS2 gene seems to represent a protection factor against the development of BCC tumours in individuals undergoing transplantation before 50 years of age, and this effect is probably abolished in elderly individuals where the additive effects of risk factor accumulation, along with a reduced DNA repair capacity exert stronger effects. Analysis of polymorphisms in the 5' regions of the mPGES-1 and PTGER1 genes did not support the hypothesis that variants in these regions could play a major role in NMSC predisposition.

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