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Multidrug Resistance in Oral Cancer

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Tobacco associated oral cancer remains an enigma for oncologists due to their poor or partial response to chemotherapy, thereby leaving surgery and radiation as the mainstay treatment modalities. The poor clinical response of oral squamous cell carcinomas may be attributed to intrinsic or acquired multidrug resistance (MDR). Cellular drug resistance in oral cancer appears to be mediated by several mechanisms acting at different steps of the cytotoxic action of the drug: from a decrease of drug uptake in the cell to the abrogation of apoptosis induced by it. These mechanisms include (i) activation of transmembrane proteins effluxing different drugs from the cells (P-glycoprotein and MRP-1); (ii) activation of cytosolic drug detoxification system-glutathione-S-transferase pi; (iii) alterations of proteins involved in regulation of cell cycle and apoptosis: p53 and bcl-xL and (iv) activation of DNA repair enzyme-DNA topoisomerase II. Knowledge of biochemical mechanisms of MDR may pave the way for designing effective strategies for its circumvention and might lead to the identification of candidate molecular markers for predicting the response of oral tumours to anticancer drugs. Use of DNA microarrays for genome wide screening for markers of drug resistance may lead to identification of new molecular targets for rational designing of molecular therapeutics for combating drug resistance.

Key words: **Multidrug resistance, Human oral cancer, p-glycoprotein, GSTpi, Anticancer drugs**

Introduction

Drug resistance is the failure of primary or secondary tumours to respond to anticancer agents. Multidrug resistance is the protection of cells against several drugs differing in chemical structure and mechanism of action. MDR is one of the major causes of failure of chemotherapy in the management of human cancers. MDR can occur at any of the several steps between the administration of a drug to the patient and the desired effect of tumour cell death. The putative sites of drug action include: (i) penetration of the drug into the tumour microenvironment; (ii) uptake of the drug by the target cells; (iii) activation or changes in the metabolism of the drug in the cell; (iv) interaction with cellular targets; (v) subsequent signalling events triggering cell cycle arrest or cell death, apoptosis. Thus, MDR may arise due to factors that impair drug delivery to tumour cells, or genetic and epigenetic alterations affecting drug sensitivity that arise in the cancer cell itself.

An impaired delivery of a drug to the tumour cells may result from poor absorption of orally administered drugs, increased drug metabolism or increased excretion, resulting in reducing the level of drug in circulation and its decreased diffusion into the tumour mass (Jain 2001a, Pluen et al. 2001). The tumour vasculature and pressure gradient for effective drug delivery to the tumour are also important determinants of drug pharmacokinetics (Jain 2001b). *In vitro* experimental models, drug resistant cancer cells obtained by exposure of cancer cells to anticancer agents and selection of drug resistant clones, have been extensively used to delineate the molecular mechanisms involved in MDR (Chen et al.1986). However, some cancer cells that are sensitive to anticancer drugs as monolayer cells in culture have been found to become resistant when transplanted into animal models (Green et al. 1999).

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These findings suggest that intercellular environmental factors, such as extracellular matrix, cell to cell interaction and tumour geometry might also play a role in drug resistance. Resistance of tumour cells to chemotherapy has been shown to be modulated by the three dimensional architecture of spheroids and solid tumours (Durand & Olive 2001, Pluen et al. 2001). The microenvironment of rapidly growing tumours is associated with increased energy requirement and diminished vascular supply, resulting in focal areas of hypoxia. Recent data indicate that *mdr1* gene is hypoxia responsive, induced by hypoxia-inducible factor-1 (HIF-1) and suggest that hypoxia elicited Pgp expression may be a pathway for resistance of some tumours to anticancer drugs (Comerford et al. 2002). In addition, host tumour interactions may also contribute to clinical drug resistance in cancer patients. Knowledge about this type of drug resistance and its role in clinical oncology is meagre. However, this review will focus on cellular mechanisms of drug resistance in oral cancer; the problems of drug pharmacokinetics will not be considered.

Knowledge of different mechanisms adopted by cancer cells to evade the cytotoxic effects of chemotherapeutic drugs is of immense importance in designing novel strategies for circumvention of MDR in cancer treatment. Identification of molecular alterations in drug resistant cells may enable the use of molecular markers of drug resistance to predict the clinical response to anticancer drugs. Study of biomarkers of drug resistance may help to predict which patient is likely to benefit from chemotherapy. Advances in genome wide screening for markers of drug resistance and targeted drug design may pave the way for effective management of cancer. MDR in human cancers has been extensively studied at the cellular and molecular levels and exhaustively reviewed (Johnstone et al. 2000, Stavrovskaya 2000, Gottesman et al. 2002 and references therein). Hence, this review primarily focusses on drug resistance in oral cancer. It comprises of an overview of the state of the art knowledge of cellular mechanisms of multidrug resistance in human oral cancer, with special emphasis on its detection and circumvention for effective disease management.

Oral Cancer: An Enigma

Cancer of the oral cavity and pharynx ranks sixth overall in the world for both genders (Johnson 2000). Globally, the annual age adjusted incidence rate for oral cancer in males is 6.42 per 10^5 and in females is 3.27 per 10^5 (Globocan 2000). Oral cancer is the commonest form of cancer among Indian males; the age adjusted incidence rate is 12.84 per 10^5annually and is the third most common malignancy in females 7.49 per 10^5 (Globocan 2000). Population based 5 year survival for patients with this group of cancers are approximately 50% in United States, 45-49% in Europe and 35% in the developing countries (Sankaranarayanan et al. 2000). Epidemiological data suggest a causal association between tobacco and betel quid chewing habit, prevalent in the Indian subcontinent and the incidence of oral cancer (Jussawalla & Deshpande 1971).

Chronic exposure of the oral mucosa of tobacco or betel quid chewers to genotoxic constituents of these masticatories may cause defects in the ability to metabolize these carcinogens, repair DNA damage, control cell signalling and cell cycle checkpoints, processes fundamental to cellular homeostasis (Baden 1987, Park et al. 1987, Trivedi et al. 1993, Idris et al. 1996, Hutter & Sinha 2001, Das & Nagpal 2002, Ralhan & Kaur 2003). The defects in these cellular mechanisms culminate in the development of clinically distinct precancerous lesions, such as erythroplakia, leukoplakia or precancerous condition oral submucous fibrosis; 5-10% of these lesions progress to malignancy over a period of 2-10 years (Daftary et al. 1991). Thus, the development of tobacco related oral cancer is a multistep process (Ralhan 1999).

Clinical Management of Oral Cancer

The term oral cancer generally refers to squamous cell carcinoma (SCC) of oral mucosal origin, which accounts for more than 90% of all malignancies of this location (Barasch et al. 1998). The mainstay of current therapy for oral cancer is surgery or radiation. The 5 year survival rates of oral SCC patients are similar for both modalities and a decision is made on the basis of tumour's site and size, nodal stage, and histologic findings (Ord & Blanchaert 2001). Despite advances in therapy,

long-term survival of oral cancer patients has not improved significantly (McCann et al. 2000). The prognosis of oral SCC patients is adversely influenced by the development of second primary tumours (SPTs). The incidence rate of SPTs is 10-35%, depending upon both the site of the first primary tumour & the age of the patient (Jovanovic et al.1994). The role of chemotherapy in the management of oral SCC remains to be clearly defined. Use of cytotoxic agents alone for oral SCC has shown no success in improving life expectancy in these patients (Harari et al. 1997). Chemotherapy is being used as adjuvant or neoadjuvant therapy for management of oral cancer. It is the only systemic therapeutic approach for treating metastatic disease. Studies of concomitant or alternating chemo-radiation have reported significant therapeutic benefits for patients with advanced disease (stages III and IV) (Barasch et al. 1998, Ord & Blanchaert 2001). Combination therapies have been shown to be superior in both overall survival & disease free survival, despite severe side effects during treatment. A comprehensive review of all prospective randomized trials of chemotherapy for head and neck cancer (inclusive of the oral cavity) did not show evidence of increased survival when chemotherapy alone was used (reviewed in Fu 1997). Chemotherapy is usually combined with radiation. The anticancer drugs used for treatment of oral cancer include cisplatin, carboplatin, 5-fluorouracil (5-FU) and the taxanes (paclitaxel and docetaxel) (table 1). The taxanes and 5-FU have also been shown to act as radiation sensitizers. Concurrent chemo-radiation has been shown to increase survival rates in patients with head and neck cancer (El-Sayed & Nelson 1996). Most series have combined all head and neck cancer sites; therefore it is difficult to interpret data for the oral cavity alone. In a recent study of predictors of response and survival after concurrent chemotherapy and radiation for locally advanced SCC of the head and neck, only 17 of 111 (15%) cases involved the oral cavity (Suntharalingam et al. 2001).

In a recent study dihydropyrimidine dehydrogenase (DPD) mRNA levels have been shown to correlate with the response to 5-FU-based chemo-immuno-radiation therapy in human oral squamous cell carcinomas. These observations suggest that intra-tumoural levels of DPD mRNA may predict tumour response to 5-FU-based chemotherapy in oral cancer patients (Hoque et al. 2001).

Drug Resistance in Oral Cancer

A large number of oral cancer patients show poor or partial response to anticancer drugs, so that drug

Table 1 ***Anticancer drugs used in oral cancer therapy: Mechanism of action***

Families of Anticancer Drugs	Drugs	Mechanism of Action	Drug Targets	References
Drugs derived from plants	Taxanes: Paclitaxel, Docetaxel	Polymerization of microtubules, damage to mitotic spindle	Cytoplasmic, microtubules, mitotic spindle	Eckardt et al. 1999, Eckardt et al. 2002, Kovacs et al. 2002, Myoung et al. 2001, Ruzich et al. 2002
Platinum compounds	Cisplatin (CDDP), Carboplatin	DNA binding, DNA strand breaks, DNA crosslinking	DNA	Cutilli et al. 1998, el Attar et al.1997, Elattar et al. 2000, Hemmer et al. 1994, Ita & Murakami 1998, Kanata et al. 2000, Masumoto et al. 1995, Matsumiya et al. 2001, Ohtani et al. 2000, Wang et al. 1989
Antimetabolites	Methotrexate (MTX), 5-Fluorouracil (FU)	Inhibition of enzymes involved in synthesis of DNA and RNA	Dihydrofolate reductase, Thymidylate synthetase	Desprez et al. 1966, Desprez et al. 1970, Helpap et al. 1977, Hoque et al. 2001, Koura 1970, Ohtani et al. 2000

resistance remains an enigma for oral oncologists. The molecular mechanisms causing resistance to chemotherapeutic agents in oral cancer patients are not clearly understood. As observed in several human cancers clinical drug resistance in oral cancer may also be intrinsic or acquired.

Intrinsic Drug Resistance

Oral cancer patients with intrinsic (innate or *de novo*) drug resistance fail to respond or show poor response to administration of the first course of anticancer drug treatment. There is increasing evidence that Pgp-mediated MDR is likewise developing in the multicellular context of tumour tissues, without previous treatment with anticancer agents (Dhar et al. 1995, Pilarski & Belch 1995, Oda et al. 1996, Wartenberg et al. 1998a, Wartenberg et al. 1998b). Intrinsic drug resistance may be attributed to the activation of *mdr1* gene product, Phosphoglycoprotein (Pgp), due to early exposure of an individual to Pgp substrates or chemicals such as some carcinogenic tobacco constituents (Lo Muzio et al. 2000), or low levels of reactive oxygen species (ROS) (Wartenberg et al. 2001). Recently it has been pointed out that the expression of Pgp may be redox-regulated (Stein et al. 1997, Deshpande et al. 2000, Wartenberg et al. 2001). Low levels of ROS have been shown to induce the expression of *mdr1* gene (Wartenberg et al. 2001). Elevation of intracellular ROS was demonstrated to downregulate Pgp expression via the activation of receptor tyrosine kinase signalling pathways, which resulted in the phosphorylation of Mitogen activated protein kinase (MAPK) members and mitogenic stimulation (Wartenberg et al. 2001). Furthermore, circumvention of MDR by modulation of the intracellular redox state by BSO or other ROS-generating agents *in vitro* suggested its probable utility *in vivo* to promote efficacy of chemotherapeutic regimen in anticancer treatment. On the other hand it has been reported that high levels of ROS result in severe cellular oxidative stress and increased the expression of MRP-1 (Yamane et al. 1998) and MDR-1b (Ziemann et al. 1999) genes.

Acquired Drug Resistance

In acquired drug resistance, oral cancer patients initially respond to a drug, but eventually exhibit resistance not only to this drug, but also to a variety of other drugs, which may or may not be structurally related to it. The broad spectrum resistance to drugs is termed as multidrug resistance (MDR). This cross-resistance of oral tumours to multiple chemotherapeutic drugs poses a major impediment to successful cancer chemotherapy (Chen et al. 1986, Mese et al. 1998). The MDR phenotype is characterized by the resistance of tumour cells to a spectrum of structurally diverse compounds with discrete subcellular targets including plasma membrane, cytosol and nucleus. Acquisition of the MDR phenotype in human cancer cells may result from one or more mechanisms acting simultaneously, thus multidrug resistance may be multifactorial. Cancer cells are genetically heterogenous. Tumour cells exposed to chemotherapeutic agents will be selected for their ability to survive and grow in the presence of cytotoxic drugs. These tumour cells are likely to be genetically heterogeneous because of the mutator phenotype. Therefore, in any population of tumour cells exposed to cytotoxic drugs, more than one mechanism of MDR can be present. This phenomenon is known as Multifactorial Multidrug Resistance (Lehnert 1996, Larsen et al. 2000, Shain & Dalton 2001).

Cellular Mechanisms of Multidrug Resistance in Oral Cancer

Oral cancer cells may show resistance to anticancer drugs in a myriad of ways illustrated in figure 1. Drug resistance can be mediated by different mechanisms operating at different steps of the cytotoxic action of the drug: from a decrease of drug accumulation in the cell to the abrogation of cell death (apoptosis) induced by the anticancer agent. The most well studied mechanisms are:

I. Activation of transmembrane proteins effluxing anticancer drugs from the plasma membrane:

Increased expression of Multidrug Transporters: P-glycoprotein (Pgp) and, Multidrug Resistance-associated Protein (MRP).

II. Activation of cytosolic enzymes of the drug detoxification systems

a. Gluthathione S-transferase pi (GST pi)

b. Cytochrome P-450 hydroxylase (CYP1B1)

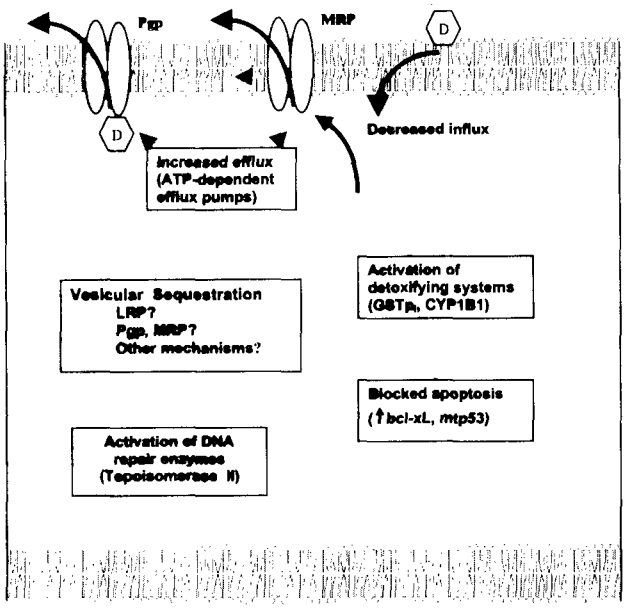

Figure 1 Cellular mechanisms of drug resistance. Oral cancer cells become resistant to anti-cancer drugs by several mechanisms. These include: (i) increased activity of ATP-dependent multidrug transporters such as P-glycoprotein and MRP-1; (ii) decreased influx of the drugs; (iii) activation of detoxification enzymes such as CYP1B1, GSTpi; (iv) repair of drug induced DNA damage, DNA topoisomerase II; (v) disruption of apoptotic pathways caused by alterations in genes involved in controlling apoptosis, bcl-xL, p53; (vi) vesicular sequestration of drugs.

Abbreviation *D*, Drug

III. Alterations of the genes and proteins involved in regulation of apoptosis

a. Increased levels of Bcl xL

b. Mutations in p53 tumour suppressor gene

IV. Factors acting at the nuclear level

Activation of DNA repair enzymes: DNA topoisomerases II

These mechanisms may occur independently or in combination, thereby conferring MDR in patients exposed to structurally and functionally diverse anticancer agents.

Multidrug ABC Transporters

Resistance to natural product hydrophobic drugs also known as classical MDR, often results from expression of ATP-dependent drug efflux pumps. These pumps exhibit broad drug specificity and belong to a family of ATP-Binding Cassette (ABC) transporters that share sequence and structural homology. Forty eight human ABC genes have been identified so far (reviewed in Gottesman et al. 2002). These have been divided into 7 distinct subfamilies (ABCA-ABCG) on the basis of their sequence homology and domain organization (Dean et al. 2001).

P-glycoprotein

The most commonly observed indicator of MDR is overexpression of the *multidrug resistance gene (mdr-1)* or *ATP-Binding Cassette, subfamily B, Member 1 (ABCB1)* product, P-glycoprotein (Pgp). This 1280 amino acid, 170 kDa transmembrane phosphoglycoprotein serves as an ATP-dependent efflux pump (Reviewed in Gottesman et al. 2002). Pgp consists of four distinct domains. Two of these are highly hydrophobic integral membrane domains, α-helices, each of which spans the membrane six times. The other two are hydrophilic nucleotide-binding domains (NBDs) (Chen et al. 1986). In the Pgp molecule there is a large central pore, ~5 nm in diameter, which is closed on the inner (cytoplasmic) side of the plasma membrane. A gap may be present in the protein ring; this could allow substrates to access the central pore from the lipid phase. P-glycoprotein is predicted to act as a flippase with drug substrates, gaining access to their binding sites from the inner leaflet of the lipid bilayer (Romsicki & Sharom 2001). The transmembrane region binds hydrophobic drug substrates that are either neutral or positively charged, and are transported out of the cell. The transport of one drug molecule requires 2 ATP hydrolysis events, which do not occur simultaneously (Senior & Bhagat 1998). The binding of the drug to the transmembrane region has been shown to stimulate the ATPase activity of Pgp, causing a conformational change that releases the drug to either the extracellular space or the outer leaflet of the membrane, from which it diffuses into the extracellular medium (Ramachandra et al. 1998). Hydrolysis of the second ATP molecule is required for re-setting the multidrug transporter so that it can bind another drug molecule (Sauna & Ambudkar, 2000). Pgp binds to taxanes, anthracyclines, vinca-alkaloids, epipodophyllotoxins and camptothecins. This broad specificity ABC transporter extrudes a variety of cytotoxic drugs from cells, thereby lowering their intracellular concentrations and reducing their toxic effects on tumour cells

(Gottesman & Pastan, 1993). Activation of Pgp may be regulated through phosphorylation by protein kinases, e.g. protein kinase C (Hamada et al.1987, Chambers et al.1992). The drug efflux function of Pgp may be affected by membrane lipid composition (Escriba et al. 1990) and fluidity (Sinicrope et al. 1992).

Differential subcellular localization of Pgp and its relationship with increased MDR levels of tumour cells has been demonstrated (Brinkmann & Eichelbaum 2001). Tumour cells with "classical" MDR phenotype showed increased expression of Pgp localized in the plasma membrane. Recent studies showed that Pgp was also detected on the nuclear envelope and on the membranes of the cytoplasmic organelles. Cytoplasmic Pgp has been proposed to regulate the intracellular trafficking of drugs, sequestering drugs into the cytoplasmic vesicles and their transportation outside the cells (Molinari et al. 2002). Intracellular Pgp seems to represent a complementary protective mechanism of tumour cells against cytotoxic agents. A direct correlation between intracellular Pgp and intrinsic resistance has been demonstrated in human colon carcinoma (LoVo) clone, which did not express the drug transporter on the plasma membrane (Molinari et al. 2002). Pgp has also been proposed to regulate apoptosis, immune cell function, cellular differentiation, proliferation and survival (Johnstone et al. 2000).

Multidrug Resistance-Associated Proteins

The human multidrug resistance associated protein (MRP or ABCC) family comprises of seven members, ABCC1-6 and ABCC10 (reviewed in Borst et al. 2000). These proteins have the ability to transport a wide range of anticancer drugs out of cells. Their expression has been reported in many human tissues where these pumps have major physiologic functions. Their expression is increased in human cancers though their contribution to clinical drug resistance remains to be determined. The substrate specificity for MRP1 (ABCC1) for vinca-alkaloids, anthracyclines, cisplatin and taxanes seemed to be similar to that of Pgp in initial studies (Grant et al. 1994, Zaman et al. 1996). However, later studies showed that MRPs transport organic anions, such as methotrexate and neutral drugs conjugated to acidic ligands, such as glutathione (GSH) glucuronate or sulphate, whereas Pgp has a low affinity for negatively charged compounds. In addition, MRP1 (ABCC1), MRP2 (ABCC2'also known as cMOAT, canalicular multispecific organic anion transporter) and MRP3 (ABCC3, MOAT-D) may also cause resistance to neutral organic drugs that are not known to be conjugated to acidic ligands by transporting these drugs with free GSH. MRP1 (ABCC1) can cause resistance to arsenite and MRP2 (ABCC2) to cisplatin, probably by transporting these compounds in complexes with GSH (Ohga et al. 1996). Overexpression of MRP4 (ABCC4, MOAT-B) has been shown to be associated with resistance to nucleoside analogues 9-(2-phosphonyl methoxyethyl) adenine and azidothymidine used as anti-human immunodeficiency virus drugs (Schuetz et al. 1999). MRPs may have a role in resistance against nucleoside analogues used as anticancer agents (Borst et al. 2000).

ABC transporters, including MDR1 (ABCB1), Multidrug resistant associated protein 1 (MRP1 or ABCC1) and ABCG2 can confer multidrug resistance to cancer cells *in vitro*. Mitoxantrone, an anticancer drug, is a poor substrate for Pgp and MRP1. Selection for mitoxantrone resistance results in drug resistant cancer cells that produce a more distant member of ABC transporter family, ABCG2- also known as MXR (mitoxantrone resistance) gene, BCRP (breast cancer resistance protein) or ABC-P (ABC transporter in placenta) (Miyake et al. 1999, Doyle et al. 1998, Allikmets et al. 1998). Some other ABC transporters such as ABCC2 (MRP2), ABCC3 (MRP3), ABCC4 (MRP4), ABCC5 (MRP5), ABCA2 and ABCB11 (BSEP) have been shown to be capable of transporting drugs. However, their role in drug resistance remains to be determined. The bile salt export protein (BSEP or ABCB11) or SPGP (sister of Pgp), is expressed at high levels in liver cells and in transfection experiments it has been shown to confer low level resistance to paclitaxel (Childs et al. 1998).

ABC Transporters in Normal Cells

Many ABC transporters have been identified as drug resistance proteins, yet they are all expressed in normal tissues and transport endogenous substrates (reviewed in Gottesman et al. 2002). Pgp and MRP1 are expressed in normal intestine, liver,

kidney, testis, placenta, blood brain barrier (BBB), stem cells and early progenitors in normal bone marrow (cells expressing CD34 antigen). The blood brain barrier is formed by the endothelial cells of capillaries, with Pgp located on the luminal surface. It prevents the penetration of cytotoxic agents across the endothelium (Schinkel et al. 1996, Xie et al. 1999). Pgp transports toxins into the capillary lumen in the testis. Pgp is localized on the apical syncytiotrophoblast surface in the placenta, where it can protect the foetus from toxic cationic xenobiotics (Cordon-Cordo et al. 1990). Thus ABC transporters expressed in the brain, testis and placenta protect these from cytotoxins. In liver, gastrointestinal tract and kidney ABC transporters are involved in excreting toxins thereby protecting the entire organism. Pgp localized in the apical membranes of hepatocytes transports toxins into bile (Schinkel et al. 1997). In the gastrointestinal tract, Pgp is localized in apical membranes of mucosal cells, where it extrudes toxins and might have a role in determining oral drug bioavailability (Mayer et al. 1997, Lown et al. 1997, Greiner et al. 1999). In addition, Pgp has been shown to secrete intravenously administered drugs into the gastrointestinal tract (Sparreboom et al. 1997). MRP1, in constrast to Pgp, is located in the basolateral membrane of intestinal mucosal cells and transports substrates into the interstitium and the blood stream (Evers et al. 1996).

ABC Transporters in Human Cancers

ABC transporters MDR1 and MRP1 have been found to be expressed in many human cancers including solid tumours and leukemias. In some studies the expression of these transporters has been shown to correlate with response to therapy and survival (Borst et al. 2000, Gottesman et al. 2002). Pgp is the most widely studied ABC transporter in human cancers. Overexpression of Pgp has been reported in leukemias, sarcomas, osteosarcomas and solid tumours such as cancers of colon, kidney, liver, breast, head and neck, lung and ovaries (Fojo et al. 1987, Goldstein et al. 1989, Trock et al. 1997). However, the failure of these cancers to respond to drugs that are not Pgp substrates suggested that other factors may also be involved. The key question is: Does Pgp expression confer drug resistance in human cancer? Most studies that have correlated Pgp expression with clinical outcome have been single institution studies with small sample size that had insufficient power to draw reliable conclusions (van den Heuvel-Eibrink et al. 2000). Tumour heterogeneity and presence of normal tissue in tumour biopsies further complicates the assessment of Pgp expression in solid tumours. Trock et al. (1997) carried out a meta-analysis of 31 reports from 1989-1996 of *mdr1*/Pgp expression in breast cancer, wherein 41% of breast tumours expressed Pgp. Expression of Pgp increased after treatment and was associated with a greater possibility of treatment failure. However, considerable inter-study variation was observed in this meta-analysis as well as in subsequent studies thereby hampering the confirmation of a conclusive role of Pgp in breast cancer (Schneider et al. 2001). Hence, larger multi-institutional studies are required for unequivocal confirmation of role of Pgp in conferring MDR in human cancer.

Methods to Detect Expression of Pgp/*Mdr1* in Human Oral Cancer

Several methods have been used to measure the cellular expression or function of Pgp/ *mdr1*. These methods include use of specific antibodies for detection of Pgp protein by immunohistochemistry (Ralhan et al. 1997) and flowcytometry (Jain et al. 1997). Alternatively, functional assays have been used for detection of Pgp that include measurement of drug retention (Kawase et al. 2002) or drug efflux (Sudimack et al. 2002). The molecular method using reverse transcription – polymerase chain reaction has also been employed for assessment of *mdr-1* expression in oral cancer (Cho & Kim 2001).

The major problem with designing a clinical study that provides statistically valid results is that methods for detecting Pgp expression are imperfect, primarily due to lack of specificity of many commercially available anti-Pgp antibodies. The problem is further complicated by the use of different methods to quantify Pgp expression, to control for tumour heterogeneity, and to account for the presence of normal tissue in tumour biopsies.

A set of consensus recommendations taking into account the advantages and disadvantages of the

various methods available to detect and quantitative MDR associated with Pgp and *mdr-1* in human cancers has been reported (Beck et al. 1996). Immunohistochemical methods have the inherent advantages of providing information on the cell type and subcellular localization of the protein, in addition to giving semiquantitative information on Pgp expression. However, because some of the available antibodies cross-react with non–Pgp epitopes, it has been recommended that at least 2 antibodies be used, one recognizing an extracellular and one an intracellular epitope of Pgp. For flow cytometrical detection of Pgp using monoclonal antibodies, it is recommended that one uses antibodies that recognize extracellular epitopes, because their use in flow cytometry allows for correlation with other cell-surface antigens by multiparameter flow cytometry and for flow cytometrical measurement of drug/dye efflux by the intact cells. All antibody controls should be isotype matched. When using methods for measurement of drug or dye efflux, it is recommended that they be used in parallel with measurements of Pgp (by flow cytometry) to distinguish between Pgp– and non-Pgp–mediated drug effluxes.

PCR-based methods for *mdr1* detection and measurement represent the most specific and the most sensitive of all methods for measurement of Pgp/*mdr1*. On the other hand, because *mdr1* expression is not restricted to neoplastic cells, detailed information regarding the purity of the samples undergoing analysis must be provided. Control experiments should be carried out in parallel with each cDNA synthesis and PCR reaction, using well-characterized control cell lines (Norgaard et al. 1998). Qualitative PCR determinations are of limited value, and quantitative PCR is subject to a number of methodological pitfalls. These include quality control of RNA, selection of appropriate control household genes, asymmetrical amplification of construct versus native cDNA in competitive PCR, and unclear significance of low or very low *mdr1* expression. Recently, quantitation of PCR products has been made considerably easier by the introduction of fluorochrome-labeled primers. Coupled with real-time measurements of amplification, a predefined threshold may be employed to distinguish levels of mdr1 RNA in the different samples.

P-glycoprotein Expression in Oral Cancer

In experimental models, the multidrug resistance phenotype is often accompanied by the expression of the *mdr1* gene product, Pgp. Drug resistant cell lines have been established by growing oral cancer cells in the presence of anticancer drugs. The drug resistant clones selectively grown from heterogenous tumour cell population serve as *in vitro* experimental models for studying the molecular mechanisms implicated in drug resistance (Wang 1993, Urade et al. 1994, Zhang et al. 1999). The biological characterization of vincristine-resistant cell line established from human oral SCC showed varying degree of resistance to adriamycin, cisplatin, methotrexate and 5-fluorouracil (Zhang et al. 1999). Overexpression of *mdr-1* gene in these cells suggested that the MDR phenotype of this cell line is attributed to Pgp expression (Zhang et al. 1999).

The role of ABC transporters associated with drug resistance in oral cancer is not clearly defined. The specific questions that need to be addressed in order to gain insight into the role of Pgp in clinical drug resistance in oral cancer are: Is Pgp expressed in oral SCCs? If yes, what is the frequency of its expression in oral tumours? Is Pgp expression associated with clinical drug resistance? What is the potential functional relevance of Pgp expression in oral tumours with respect to response to chemotherapy and predicting prognosis? Few studies have been undertaken to assess the expression of Pgp in oral SCCs and its relationship with prognostic parameters, response to therapy and survival (table 2). All these studies reported Pgp expression in oral carcinomas, though the frequency of Pgp expression varied among the different studies (table 2). Analysis of Pgp expression in human oral tissue specimens from different stages of oral tumour development and progression showed differential expression of this protein in oral normal, dysplastic, primary and recurrent malignant tissues (Jain et al. 1997, Ralhan et al. 1997).

The commonly prevailing habit in India is to keep the tobacco or betel quid at a particular site in the mouth repeatedly for prolonged time period. Chronic exposure of this particular site for several years to the carcinogenic constituents culminates in accumulation of genotoxic insults, resulting in the development at this site of a leukoplakic lesion,

Table 2 *P-glycoprotein expression in Oral Cancer*

Authors	Survival	Patient N	P/R	Pgp Expression N	%	Prognostic Parameters	Controls N	%	CT Response	Method	Ab Used
Narayanan et al. 1994	ND	13	R	13	100	NA	3/16	19	Sx,+50-60Gy, CMV	IHC, WB	[a]MRK-16
Rabkin et al. 1995	Decreased survival	33	P	33	100	NA	ND	ND	UT	IHC	JSB1, C494
Jain et al. 1997	ND	13 12 18	D P R	13 12 18	29.46 67.93 81.23	NA	12	26	UT UT Sx,50-60 Gy, CBV	FC	MRK-16
Ralhan et al. 1997	ND	32 50 31	D P R	16 42 31	50 84 100	D-grade T-sage	7/41	17	UT UT Sx,50-60Gy, RT, CBV	IHC	[a]C219
Xie et al. 1998, 2000	ND	40	P	25	62.5	ND	ND	ND	UT and T	IHC	MRK-16
Ng et al. 1998	ND	20 36	P P	1 23, 26	2.6 63.9, 72.2	ND	ND	-	UT Post RT	IHC	C219, C494
Ralhan et al. 1999	Decreased DFS	35 100	L P	25 81	71 81	-	4/70	6	UT	IHC, FC	[a]C219, MRK-16
Swain et al. 1999	ND	84 46	P R	50 43	60 93	ND	ND	-	UT 50-60Gy, CMV	IHC	[a]C219
LoMuzio et al. 2000	-	30	P	24	80	ND	4/6	66.6	UT	IHC	[b]
Warnakulasuriya et al. 2000	Decreased DFS and OS	21	P	4	20	ND	ND	ND	UT	IHC	JSB-1, MAb 9011
Cho and Kim 2001	-	52	P	0.37	-	ND	22/22	0.40	UT	RT-PCR	-
Uematsu et al. 2001	-	37	P	12	32	ND	-	-	UT	IHC	-
Soni et al. 2002	Decreased Survival	40	P	27	68	LNM	NA	-	UT	IHC	[b]C219

ND, not determined; *UT*, untreated; *D*, dysplasia; *L*, leukoplakia; *P*, primary tumour; *R*, recurrent tumour; *DFS*, disease free survival; *OS*, Overall survival; *IHC*, immunohistochemisry; *WB*, western blot; *RT-PCR*, reverse transcription-polymerase chain reaction; *CMV*, cisplatin, methotrexate and vincristine; *CBV*, cisplatin, bleomycin and vincristine; *N*, number; *CT*, chemotherapy; *LNM*, lymph node metastasis; *Sx*, Surgery; *RT*, radiotherapy; *FC*, flow cytometry; [a] Frozen sections; [b]paraffin sections.

which may undergo malignant transformation (Daftary et al. 1991). Expression of Pgp in the oral cavity of habitual betel and tobacco consumers may suggest a role as a xenobiotic transporter in this tissue (Ralhan et al. 1999). Lo Muzio et al. (2000) demonstrated Pgp positivity in oral normal epithelial cells of tobacco smoker patients, and in the differentiated areas of oral SCCs, whereas negativity or zonal positivity was shown in undifferentiated areas of the tumours suggesting that activation of the *mdr-1* gene or selection of intrinsically multidrug resistant neoplastic cells may occur at early stages of tumourigensis of oral cancers, before the real evidence of cellular transformation. Thus, the contact of oral epithelial cells with possible chemical carcinogens, such as those of tobacco smoke, may induce activation of *mdr-1* gene. The possibility that Pgp can act as an efflux pump-for compounds in diet or encountered in the environment has been suggested (Bradley et al. 1990). Yeh et al. (1992) proposed that cellular burden of certain carcinogens [benzo (α) pyrene] may be mitigated by Pgp, suggesting its role in cellular defense against carcinogens. It has also been suggested that *mdr-1*/Pgp expression might be associated with induction of other drug detoxification mechanisms (Lo Muzio et al. 2000). It may also be possible that exposure to tobacco carcinogens induces a cellular stress response that co-induces several stress and detoxification mechanisms. This response could include increased expression of heat shock proteins, glutathione-S-transferases and superoxide dismutase. The activity of some DNA repair processes including topoisomerases may also be altered (Lo Muzio et al. 2000).

The expression of Pgp in oral dysplasias suggests that alteration in Pgp expression correlates with early events in oral oncogenesis. Detection of Pgp protein in untreated primary oral tumours suggests that these tumours are likely to exhibit intrinsic drug resistance and could possibly be the rationale for the limited efficacy of anticancer drugs in the management of oral cancer (Ralhan et al. 1997). The level of Pgp expression in oral SCCs showed positive correlation with resistance to vinblastine in *in vitro* assays, suggesting that Pgp expression in untreated primary or recurrent oral SCCs may serve as a predictive biomarker for clinical response to chemotherapy (Kaur and Ralhan, unpublished observations). Pgp expression was significantly increased in recurrent oral SCCs as compared to untreated primary oral tumours suggesting that in addition to the innate drug resistance exhibited by primary oral SCCs, acquired MDR in recurrent tumours may add to the complexity of chemoresistance in oral cancer (Ralhan et al. 1997).

Association of Pgp Expression with Prognosis, Recurrence and Survival

Clinical studies demonstrated that Pgp positivity in oral SCCs correlated with aggressive tumour behaviour (Ralhan et al. 1997, Warnakulasuryia et al. 2000). Increased Pgp expression has been observed in SCC of the base of tongue in well differentiated areas of the tumours as well as in tumours with diploid DNA content and correlated with decreased survival (Rabkin et al., 1995). Few studies have focussed on clinical specimens to investigate the impact of coexpression of p53 and Pgp proteins on disease prognosis. Patients with oral SCCs harbouring concomitant expression of p53 and Pgp proteins had shorter disease free survival (recurrence/metastasis) and may account for the aggressive nature of the tumour and poor prognosis (Ralhan et al. 1999). Recently, concomitant expression of Ets-1, p53 and Pgp proteins in oral SCCs was reported to adversely affect the clinical course of this disease. In univariate analysis, the expression of each of these proteins, Ets-1, Pgp and p53 was significantly associated with shorter disease free survival of oral cancer patients. Pgp however, emerged as the most significant adverse prognosticator in multivariate analysis in this cohort of oral cancer patients (Soni et al. 2002).

Association of Pgp Expression with Response to Chemotherapy

The data of Pgp expression in oral SCC in several studies (table 2) suggest that overexpression of Pgp protein may constitute a hallmark of potentially more aggressive phenotype for oral cancer and a rapid method for pre-screening tumours for a constitutive MDR to plan effective treatment strategies (Xie et al. 1998). A correlation between Pgp expression and clinical MDR of squamous

carcinoma in oral and maxillofacial region was demonstrated in Chinese population (Xie et al. 1998, 2000). Pgp expression was significantly higher in post-chemotherapy group than in the group which did not receive chemotherapy. The hallmark of this study was the correlation of Pgp expression with clinical response to chemotherapy with 75% accuracy rate of prediction. In Japanese population, Pgp related MDR in salivary gland adenocarcinomas was shown to be an inherent phenotype caused by both high levels of Pgp induction and activated Pgp production during vincristine treatment (intrinsic and acquired MDR), while that in squamous cell carcinoma was an acquired phenotype chiefly caused by induction of Pgp (Uematsu et al. 2001). In another study, oral cancer patients who had undergone radiotherapy showed increased Pgp expression and a lower rate of response to chemotherapy suggesting that increase in Pgp expression was proposed to likely attribute MDR phenotype to the tumour cells and may affect the efficacy of subsequent or concurrent chemotherapy. It may also explain the lower rate of response to chemotherapy among oral cancer patients who have previously had radiotherapy (Ng et al. 1998). Pgp and p53 expression were shown to be significant prognostic markers in advanced head and neck cancers (including oral and oropharynxgeal cancers) treated with chemo-radiotherapy; Pgp/p53 expression was suggested to be useful in stratifying patients into groups with favourable and unfavouraqble prognosis (Warnakulasuryia et al. 2000).

Determination of *p53* gene status in tumour cells may help to predict response of tumours to chemotherapy in oral and maxillofacial SCCs (Cutilli et al. 1997). *p53* mutations and their relationship with resistance to neoadjuvant therapy (three 5-FU-cisplatin cycles) was analyzed in a prospective study. Patients with *p53* mutations were chemoresistant and showed the tendency to a poor prognosis underscoring the possibility to utilize *p53* as a prognostic and chemoresponse marker for the evaluation of advanced oral cancers (Cutilli et al. 1998). However, most studies that correlate Pgp expression with clinical outcome have been small, single institution studies. Large multi-institutional studies using uniform protocols for Pgp assay are warranted to unequivocally define the role of Pgp in clinical drug resistance in oral cancer. Furthermore, the failure of oral tumours to respond to drugs that are not Pgp substrates, such as cisplatin, methotrexate and 5-fluorouracil suggest that other factors may also be involved in clinical drug resistance.

Mechanism of Activation of mdr-1/Pgp

The transcriptional regulation of *mdr-1* is complex. The mechanisms by which xenobiotics might modify the physiological *mdr-1* expression, as well as the possible role of oncogenes in dysregulation of this gene are currently under investigation (Labialle et al. 2002). Studies of genes and signalling pathways involved in regulation of *mdr-1* promoter (linked to a reporter gene) into cells showed that p53, ras, raf, Ets-1, c-jun, c-fos and retinoic acid receptors (RAR-α and RAR-β) influence *mdr-1* activity (reviewed in Stavarovskaya 2000, Labialle et al. 2002).

Activation of *mdr-1* gene in cancers derived from tissues not normally expressing Pgp may be due to the influence of cellular oncogenes and tumour suppressor genes altered during cancer progression. The wild type p53 represses the *mdr-1* gene, whereas mutational inactivation of p53 repression functions may lead to increase in *mdr-1* gene expression. The increased *mdr-1* in tumours may also be due to enhanced transactivation of mdr-1 by mutant p53 (Thottassery et al. 1997). Chin et al. (1992) showed that *ras* and mutant *p53* can activate the *mdr-1* promoter. The direct binding of p53 to the *mdr-1* promoter is still debated. It is possible that p53 may modulate its effect on *mdr-1* by interaction with other factors such as SP-1, NF-Y, NF-IL6 or C/EBP β or AP-1, all of which bind the *mdr-1* promoter (Labialle et al. 2002). Alternatively, mutant p53 may increase Pgp expression by cooperation with protooncogenic Ets-1 factor, which binds its element located from (-69 to -63 bases) in the *mdr-1* promoter (Sampath et al. 2001). The CEBPβ (NF-IL6) usually expressed at low levels in most tissues and rapidly induced in response to inflammatory cytokines (IL-1, IL-6, TNF), retinoic acid and lipopolysaccharides has been shown to activate the *mdr-1* gene (Combates et al. 1994).

TCF4, a Member of the Ternary Complex Factor (TCF) family, forms a complex with β-catenin which as been reported as an *mdr-1* activator in colorectal carcinogenesis (Yamada et al. 2000).

Recently, the *mdr-1* promoter has been shown to have a steroid xenobiotic receptor (SXR) element (-7852 to -7837 sequence), which binds a pregnane xenobiotics receptor/retinoid xenobiotic receptor α (PXR/RXRα) heterodimer, that activates *mdr-1* transcription in response to xenobiotic inducers such as rifampin, nifedipine, RU486 and dexamethasone (Geick et al. 2001). The SXR family of nuclear receptors has been proposed to coordinate drug metabolism via cytochrome P450 genes (CYP 3A4 and CYP 2 C8) and drug efflux via *mdr-1*, in addition to regulation of *mdr-1* in SXR-expressing tumours (Synold et al. 2001).

Using human oral squamous cell carcinoma cell lines specific *p53* mutants were shown to activate the *mdr-1* promoter and cotransfection of *H-ras* and *mutant p53* synergistically activated the *mdr-1* promoter in transient transfection assays (Ralhan et al. unpublished data). Mutations in *p53* and *ras* have been observed in oral malignancies in Indian population (Saranath et al. 1991, Ralhan et al. 2000). Taken together with the *in vitro mdr-1* transfection studies it may be proposed that activation of the *mdr-1* gene in oral tumours may be partly under the control of mutant p53. Furthermore, cytoplasmic sequestration and functional inactivation of p53 protein due to binding to cellular proteins such as MDM2 (Agarwal et al. 1999) or 70-kDa heat shock protein (HSP70) (Kaur et al. 1994, 1998) or due to HPV infection, in oral cancers might be predicted to lead to de-repression of *mdr-1*, resulting in the expression of Pgp in untreated primary oral SCCs.

Another possibility to account for the up-regulation of Pgp is the hypomethylation of the *mdr1* promoter (Nakayama et al. 1998). *mdr1* up-regulation has also been reported after gene rearrangements, resulting in partial deletion of the *mdr1* gene and expression of a deregulated fusion gene (Mickley et al. 1997). Recently, it has been assumed that the extent of Pgp expression may be influenced by structural variations of the *mdr1* gene (Mickley et al. 1998). Furthermore, Hoffmeyer *et al.* (2000) demonstrated multiple sequence variations in the *mdr1* gene in a Caucasian population. They identified 15 polymorphisms of the *mdr1* gene with various frequencies. The authors described a noncoding sequence change in exon 26 of the *mdr1* gene at a wobble position as being significantly correlated with altered enterocyte Pgp expression and function. As determined by Cascorbi *et al.* (2001), the three most frequent SNPs in the Caucasian population are located in exons 12, 21, and 26 (positions 1236, 2677, and 3435). Allelic variants of the *mdr-1* gene have been shown to influence Pgp protein expression and function in healthy volunteers. Moreover, it was shown in healthy volunteers that these changes are in linkage disequilibrium and may therefore be associated with transcriptional regulation of *mdr1* mRNA (Kim et al. 2001).

Functionally relevant polymorphisms in the *mdr1* gene are likely to be present among acute myeloid leukemia (AML) patients and may have an impact on response to antileukemic drug treatment. If *mdr1* polymorphisms have an impact on Pgp expression, drug efficacy, in terms of eradicating leukemic blasts may also be altered. Furthermore, the related alterations of single nucleotide polymorphisms (SNPs) in Pgp expression pattern may change pharmacokinetic profiles of the drugs used to treat this disease, with a potential for enhanced toxicity or possibly suboptimal drug levels.

In a recent study, 405 AML patients were investigated for somatic genotypes of the three most frequent single nucleotide polymorphisms (SNPs) in exons 12, 21, and 26.The homozygous wild-type alleles of all 3 SNPs demonstrated both lowest *mdr1* values and significantly decreased overall survival with a high probability of relapses. Thus, allelic variants of the *mdr1*gene may influence therapy outcome by additional mechanisms, different from Pgp expression on AML blasts, possibly involving pharmacokinetic effects of Pgp (Illmer et al. 2002). Currently, it is not known in which way *mdr1* polymorphisms influence gene expression and protein function with one exception. In exon 21, the *C* variant of the *mdr1* gene is responsible for an altered *mdr1* gene product. This polymorphism accounts for an alanine to serine substitution at codon 893 of Pgp and is responsible for an altered functional activity of the protein (Kim et al. 2001).

Multidrug Resistance Associated Protein (MRP1 or ABCC1) in Oral Cancer

Multidrug Resistance Associated Protein (MRP1 or ABCC1) is a member of the family of ABC

transporters (Cole et al. 1992). MRP1 is similar to Pgp in structure, with the exception of an amino-terminal extension that contains 5 membrane spanning domains attached to a Pgp like core. MRP1 recognizes neutral and anionic hydrophobic natural products, and transports glutathione and other conjugates of these drugs (Leo et al. 1998). Expression of MRP1 has been analysed in clinical samples using specific anti-MRP1 antibodies by immunohistochemistry. Preliminary studies showed MRP1 expression in a subset of oral SCCs (Ralhan et al. unpublished data).

Cho and Kim (2001) analyzed the expression levels of MRP1 and GSTpi in oral SCCs using RT-PCR. Overexpression of MRP1 was observed in oral SCC tissues in comparison with normal oral mucosa. Linear regression analysis showed a correlation between the expression levels of MRP1 and GSTpi in normal oral mucosa and in oral SCCs. These results suggest that MRP1 expression may be activated during oral tumourigenesis; however its role in *de novo* drug resistance in oral SCCs remains to be determined (Cho & Kim 2001).

Drug Resistance Mediated By Activation of Cytosolic Drug Detoxification Systems

The glutathione S-transferases (GSTs, EC2.5.1.18) constitute a multigene superfamily of cytosolic enzymes that catalyse the nucleophilic addition of glutathione to electrophilic centres of a wide variety of compounds, thereby playing an important role in protecting cells from xenobiotics (Habig et al.1974, Chasseaud 1979, Mannervik 1985). These enzymes are involved in detoxification of cancer chemotherapeutic agents such as cisplatin, alkylating agents and doxorubicin (Tew 1994, Teicher et al. 1987, Dulik et al. 1986, Waxman 1990). Enhanced formation of drug-GSH conjugates by the GSTs may reduce the sensitivity of neoplastic cells to the action of these drugs, thereby providing a mechanism for the emergence of drug-resistant tumours. On the basis of their structural, physicochemical, enzymatic and immunological properties, the GSTs are divided into four classes: α, μ, π and θ (Mannervik 1985, Meyer et al. 1991). The pi (π) isozyme of GST has been associated with malignant transformation in human tumours (Kodate et al. 1986). Over-expression of GST -pi has been observed in a wide variety of human tumours relative to the normal surrounding tissue, as well as in the serum of cancer patients (Shea et al. 1988, Tsuchida et al. 1989). Glutathione S-transferase pi overexpression may be associated with malignant transformation and/or drug resistance.

Immunohistochemical analysis of GSTpi expression in betel and tobacco related oral cancers and normal oral mucosa showed that mild to moderate level of GSP pi expression is observed in oral mucosa of habitual consumers. The GST pi expression in untreated primary oral SCCs predominantly varied from mild to moderate levels, while in the recurrent tumours GSTpi level was significantly increased compared to the matched normal tissues, as well as primary tumours, suggesting that alteration in GSTpi expression may be attributed to prolonged exposure to carcinogens (Sarkar et al. 1997). Concomitant expression of Pgp and GSTpi in recurrent oral SCCs suggests that clinical drug resistance in these tumours may be multifactorial (Swain et al. 1999). However, indepth studies are warranted to ascertain whether the concomitant increased expression of Pgp and GSTpi in drug resistant tumour cells mediates resistance to anti-cancer agents or represents a response of tumours (or host) cells to drug exposure. The potential of Pgp and GSTpi to serve as biological markers for predicting response to chemotherapy remains to be evaluated.

Kobayashi (1999) estimated the usefulness of plasma GSTpi in diagnosis of oral SCC and confirmed GSTpi expression in human oral SCC. There was a significant difference between the mean plasma GST-pi levels in oral SCCs (both untreated and recurrent cases) and controls ($p<0.001$). GSTpi immunoreactivity was localized in the cytoplasm and nucleus of tumour cells. Immunoelectron microscopy showed that GSTpi was localized in the mitochondria and the inter-chromatin region of the nucleus. GSTpi has been proposed to play a role in determining the risk for oral cancer particularly among light smokers (Park et al. 1999). Nishimura et al. (1996) reported that GSTpi predicts response to cisplatin (CDDP) based

chemotherapy in head and neck cancer patients, which also included patients with oral and oropharyngeal cancers. A strong inverse correlation between GSTpi and response to CDDP was observed. GSTpi expression in oral tumours in response to CDDP may in part reflect an adaptive cellular response to stress and their intact ability to respond to cytotoxic injury from drugs. CDDP resistance may also be due to increased efflux of the drug, alterations in apoptosis regulation and increased DNA repair (Chu 1994).

Analysis of GSTpi expression in human surgical lingual specimens, fibrous polyp, mild to moderate dysplasia, severe dysplasia, carcinoma *in situ* (CIS) and SCC showed more frequent nuclear and/or basal cell staining in severe dysplasia, CIS and SCC, than in benign and mild to moderate dysplastic lesions (Li et al. 1997). However, its value in clinical application still remains unclear. Immunolocalization of p53, GSTpi and CD57 antigens in oral leukoplakia showed significant relationship between p53 and GSTpi staining in moderate or severe epithelial dysplasia. The severity of epithelial dysplasia and positive immunolabelling for GSTpi may be associated with local immune response alterations in oral leukoplakia (De Paula & Gomez, 2001).

Several different mechanisms of MDR may be acting simultaneously in oral tumour cells. Some methods for assessment of MDR are aimed at determining the effect of one particular resistance mechanism, whereas other methods determine the net effect of several (or all) resistance mechanisms operative in tumour cells. Thus, various *in vitro* drug resistance assays have been developed with the ultimate goal of predicting clinical drug resistance and of tailoring chemotherapy for individual patients (Ralhan & Kaur 1994). However, it must be noted that drug resistance associated with Pgp and GSTpi cannot completely explain chemoresistance in oral cancer patients encountered in multimodality therapy.

CYP1B1 in Drug Resistance

Anticancer drug inactivation by cytochrome P450 isozyme CYP1B1 may represent a novel mechanism of resistance, influencing the clinical outcome of chemotherapy (Rochat et al. 2001). Activation of CYP1B1 correlated with resistance to platinum based drugs in oral cancer patients. Carboplatin alone, or in combination with radiation and other chemotherapeutic agents, has been used for the treatment of oral squamous cell carcinoma. However, there are some limitations for such therapy because of inherent or acquired resistance to chemotherapeutic drugs.

Role of Key Genes Controlling Apoptosis in Drug Resistance in Oral Tumour Cells

The functional connection between p53 and action of anticancer drugs which cause direct DNA damage is apoptosis, a physiological mechanism activated by p53 for regulating cell growth, but also indispensable for the cytotoxic effects. Loss of p53 activity in oral tumour cells has been shown to determine non-activation of apoptosis and drug resistance (Cutilli et al. 1997).

Association of Pgp with Ets-1

The risk factors of particular importance in oral cancer development are tobacco related chemical carcinogens and viral infection mainly human papillomavirus (HPV). In an *in vitro* multistep oral carcinogenesis model established by sequential exposure of normal human oral keratinocytes (NHOK) to "high risk" HPV and chemical carcinogens, Kang and Park (2001) showed that introduction of the HPV genome into NHOK cells bypassed the senescence checkpoint and entered into an extended, but not immortal, life span during which telomere DNA continued to shorten. The E6 and E7 oncoproteins of HPV disrupted the cell cycle control and DNA repair in immortalized HOK and enhanced mutation frequency resulting from genomic instability. However, HPV alone failed to give rise to a tumourigenic cell population, which required further exposure to chemical carcinogens. Using this multistep oral carcinogenesis model, Itakura et al. (2000) showed that both HPV infection (loss of p53) and carcinogen (benzo(α) pyrene) stimulation are associated with altered expression of *fas-R, Bcl-2* and FAP-1, and contribute to acquisition of anti-apoptotic characteristics.

Another intriguing finding in clinical studies is the significant association between expression of Pgp and p53 proteins in oral potentially premalignant lesions, suggesting that these

alterations may be associated with early preinvasive stages of oral tumourigenesis and may be inherent to the development of the malignant phenotype. Tumour suppressor gene *p53* and the genes regulated by p53 are critical elements of the cell response to stress, including the influence of anticancer drugs. In response to cell injury, tumour suppressor protein p53 is activated, which in turn causes cell cycle arrest and DNA repair or eliminates the injured cells by apoptosis. Alterations of p53 are frequent events in human tumours resulting in impaired p53 function: the cells become unable to stop in cell cycle checkpoints or die by apoptosis after injury. Thus, functional inactivation of p53 can be *a priori* result in changes of tumour cell sensitivity to the drugs.

A large study conducted by O'Connor et al. (1997) analyzed more than 100 anticancer drugs using 60 cell lines, revealing a positive correlation between p53 status and cell sensitivity to cytotoxic agents. However, the type of the p53 influence on drug resistance depends on several parameters: on mechanisms of the action of a drug, species and tissue lineage of the cells and on the genetic changes arising during carcinogenesis and tumour progression. Cisplatin has been shown to induce p53-dependent apoptosis in tongue cancer cells by acting as a positive regulator of Bax expression, suggesting that p53 status may have predictive potential with regard to response to CDDP therapy (Kanata et al. 2000)

The Bcl-2 family of genes contributes to drug resistance by inhibition of apoptosis. The study of association of carboplatin resistance with Bcl-2 family level or p53 status in drug resistant and sensitive oral carcinoma cell lines, showed that the cell lines with mutant p53 were resistant, whereas those with wild-type p53 were sensitive to carboplatin (Noutomi et al. 2002). The resistant cell lines showed elevated levels of Bcl-xL, almost double that of sensitive line, whereas neither Bcl-2 nor Bax-± levels correlated with carboplatin-resistance. The association between the Bcl-xL level and drug resistance in two transformants (xL-3, xL-6) overexpressing Bcl-xL in the carboplatin-sensitive cell line (MIT 7) was established using the gene transfer method. Both clones showed resistance to multiple chemotherapeutic agents, including carboplatin, actinomycin D, etoposide, and mitomycin C. Moreover, the carboplatin resistant cells also showed cross-resistance to other anticancer agents. These findings suggest that Bcl-xL may function as one of the key components conferring multiple drug resistance in oral squamous cell carcinomas (Noutomi et al. 2002).

Multidrug Resistance Mediated by Alterations of Drug Targets and DNA Repair

Anticancer drugs that inhibit topoisomerases stabilize the DNA-topoisomerase complex, which is easily degraded under normal conditions. In cell lines selected for resistance to topoisomerase II-inhibiting drugs, the activity or the quantity of this enzyme are reduced (Pommier et al. 1994). Anticancer drugs such as daunorubicin, adriamycin, mitoxantrone and etoposide, exhibit this type of resistance. Notably, these drugs are also the substrates for Pgp and MRP1. Experimental studies demonstrate that combination of a drug and Pgp modifier can produce MDR mediated by topoisomerase II alterations. Therefore, this type of drug resistance may occur in cells in association with other drug resistance mechanisms. The clinical relevance of this type of MDR remains to be determined.

Drug target al.teration may occur due to an increase in the quantity of target protein in the cell. In several cultured cell lines resistant to methotrexate, increased level of dihydrofolate reductase (DHFR, target protein of methotrexate) was observed. This increase was due to the amplification of the *DHFR* gene. DNA topoisomerase II expression has recently been reported to be increased in oral SCCs (Cho & Kim, 2001). However, its role in drug resistance in oral cancer remains to be determined.

Methotrexate Resistance in Oral Cancer

The antimetabolites are clinically important anticancer drugs used in combination chemotherapeutic regimens for management of oral cancer. The cytotoxicities of antimetabolites, 5-FU and antifolates, methotrexate, stem from their ability to interfere with key enzymes in nucleic acid metabolism. Methotrexate displays

significant tumouricidal activity against oral tumours. Understanding methotrexate metabolism and its sites of action gives insight into the mechanism of methotrexate resistance. Methotrexate is used for induction or palliative treatment of advanced HNSCC including oral SCC: resistance to this drug often limits its clinical utility. The mechanisms proposed for development of methotrexate resistance include increased levels of the target enzyme dihydrofolate reductase (DHFR); and decreased level of folate transport proteins required for entry of methotrexate into cells. Screening of patient tumours for DHFR content and drug uptake may provide a basis for predicting clinical response to methotrexate (Hanson & Ferguson, 1993). However, this type of drug resistance is not multiple drug resistance.

Enhanced DNA repair is probably implicated in resistance to drugs interacting with DNA, such as, nitrosomethyl urea or platinum derivatives. Cultured cells with altered sensitivity to platinum complexes showed changes in the levels of proteins recoginizing and repairing DNA injury (ERCC1, ERCC2 and ERCC3/XPB) (Chu 1994). Though some of these cells are resistant to several drugs; however, the number of drugs to which cells are cross-resistant is not as large as in cells with Pgp-MDR. The clinical significance of this type of drug resistance in oral cancer remains to be determined.

Circumvention of Drug Resistance

Knowledge of the biochemical basis of MDR in oral cancer is of paramount importance in designing new strategies for its circumvention. The development of pharmacological agents that circumvent or reverse MDR is a promising way to overcome the obstacles in successful chemotherapy. Resistance to anticancer drugs such as doxorubicin, vinblastine, vincristine or taxol can be reversed, at least *in vitro*, by a variety of resistance modifying agents called MDR reversal modulators or chemosensitizers. Development of drug resistant oral cancer sublines provided *in vitro* experimental models for testing MDR modifiers for clinical development. The Pgp inhibitors that are used in the laboratory and in clinical trials to reverse MDR are the calcium channel blockers (verapamil, dexverapamil, nifeldipin) and the immuno-suppressants, (cyclosporine A and its derivatives such as PSC-833). The other MDR modulators include: quinidine, quinacrine, amioderone, tamoxifen, GF-902128, VX710, phenothiazines, hypotensive drugs (reserpine), antibiotics (cephalosporins, gramicidin, puromycin), steroid hormones (progesterone) and non-ionic detergents. MDR modulators often reverse multidrug resistance by competing for the transport system responsible for MDR, but overall the mechanism of MDR modulation is not well understood (Stavrovskaya 2000).

A good modulator of Pgp may be (*a*) a 'fast'-diffusing transport substrate; (*b*) non-competitive inhibitor, which is not being transported itself; (*c*) or an ATPase inhibitor. Among the best reversers of Pgp are PSC-833 and other cyclosporine analogues. All these compounds are characterized by a high affinity for Pgp, and they are all strong ATPase inhibitors. Thus, ATPase inhibitors may serve as good Pgp reversers. However, verapamil and many other Pgp transport inhibitors (probably being fast-diffusing transport substrates, and thus competitive inhibitors) are strong activators of the Pgp ATPase. An ATPase activator might deplete the resistant cell for energy due to the futile pumping of the fast-diffusing substrate. Finally, vanadate, a non-competitive inhibitor is a very efficient, albeit toxic, inhibitor of Pgp, MRP, and MXR function. Thus, vanadium compounds may provide a promising MDR reversal strategy (Colin et al. 1994, Litman et al. 2001)

Modulators such as verapamil, cyclosporine A and SP788 have been shown to modulate MDR in oral cancer cell lines *in vitro* (Hori et al. 1993, Perez et al. 1993). However, many of these Pgp modulators were found to be weak inhibitors that were toxic at high doses (Ferry et al. 1996). Clinical testing showed that it is difficult to obtain the necessary concentration in the patient's blood for most of the Pgp modulators, because of their toxicity and side effects. Clinical trials with cyclosporine A and dexverapamil have shown that several complications arise in treating cancer patients with these types of drugs. The completed

trials of reversal of drug resistance using modulators have been exhaustively reviewed (Kerr et al. 1986, Fisher & Sikic 1995, Ferry et al. 1996, Fisher et al. 1996, Bradshow & Arceci 1998). More effective and less toxic inhibitors of Pgp developed include cyclosporine derivative PSC-833 and verapamil R. However, these compounds also have no clinical success, probably due to the multifactorial nature of MDR. Anti-Pgp monoclonal antibodies as modifiers of MDR are under clinical testing. Liposomal drug formulations have been extensively tried for modulation of MDR in cancer. Liposomal vinblastine was shown to effectively circumvent MDR in human oral cancer cells *in vitro* (Kaur & Ralhan 1999) and warrant indepth investigation of their potential use in reversal of clinical drug resistance. Several trials have been conducted in AML patients to determine the efficacy of Pgp modulators. A large prospective randomized study of AML patients showed an increase in relapse free and overall survival for patients receiving cyclosporine A (List et al. 2001). Administration of cyclosporine A to patients with multiple myeloma and advanced non-small cell lung cancer showed similar responses (Sonneveld et al. 1992, Millward et al. 1993). The pharmacokinetic complications associated with Pgp inhibitors have confounded their clinical effects. This might partly be due to the fact that these modulators inhibit other proteins involved in drug metabolism, such as cytochrome P450. PSC-833 and cyclosporine A inhibit BSEP and reduce the secretion of bile salts, so they might reduce bile flow and slow hepatic excretion of anticancer agents (Bohme et al. 1993). Intense efforts have been directed towards developing Pgp modulators during the past fifteen years, yet identifying a Pgp inhibitor that can effectively reverse drug resistance in humans remains a major challenge. Several new Pgp inhibitors with fewer pharmacokinetic interactions are under development. These include XR-9576, (an anthranilic acid based drug), R-101933 (benzazepine derivative to be used as an oral Pgp inhibitor), LY-335979 (a cyclopropyl dibenzosuberane moiety) (Dantzig et al. 1996, Starling et al. 1997), OC-1440935 (substituted diarylimidazole) and GF-120918 (acridine carboxamide derivative) (De Bruin et al. 1999, Gottesman et al. 2002). A Pgp and MRP1 substrate, ^{99m}Tc-sestamibi, used in cardiac function imaging, can also be used to directly image Pgp activity in both normal and tumour tissue. Increased hepatic accumulation of ^{99m}Tc-sestamibi is regarded as a surrogate marker for effective Pgp inhibition (Peck et al. 2001). Increased ^{99m}Tc-sestamibi accumulation has been observed in tumours following administration of PSC-833 and VX-710 (Chen et al. 1997, Peck et al. 2001).

Surrogate assays have been developed to evaluate the *in vivo* efficacy of Pgp inhibitors that measure the extent of Pgp inhibition. Serum obtained from patients receiving a Pgp modulator is assayed for its ability to either reverse MDR or increase drug accumulation in a Pgp overexpressing cell line (Solary et al. 1991, Minani et al. 2001).

Alternatively, $CD56^+$ cells (a subset of circulating lymphocytes, known as natural killer (NK) cells, that express the CD56 antigen) express high levels of Pgp and actively efflux rhodamine-123 dye. This efflux is eliminated following administration of a Pgp inhibitor. Thus, $CD56^+$ cells taken from patients undergoing therapy with a Pgp inhibitor can be assayed for the efflux of rhodamine-123 (Witherspoon et al. 1996, Robey et al. 1999).

Circumvention of Cisplatin Resistance

Metallothionein (MT), a low-molecular-weight protein with high cysteine content, has selective affinity for heavy metal ions and overexpression of MT has potential against resistance for CDDP and radiation treatment. MT is related to neoplastic resistance to oncologic treatment and therefore has been studied as a prognostic factor for a variety of human malignant tumours. It has a potential against resistance for CDDP anticancer agents and radiation treatment (Muramatsu et al. 2000).

Future Trends and Conclusions

The prevailing paradigm has been that Pgp-mediated drug resistance could account for low efficacy of anticancer agents in many cancers. Few studies demonstrated that Pgp may be a

critical determinant of clinical drug resistance in oral cancer. While the ABC transporters Pgp and MRP1 will directly affect intracellular concentrations of drugs and MRP 1 will directly affect at the level of cell survival pathways, drug metabolism and drug target may also contribute to MDR. Thus cellular proteins such as GSTpi, p53, Bcl-xL and Ets-1 may also be important determinants of MDR.

Multidrug resistance in oral cancer is likely to be multifactorial and may be caused by a myriad of independent or interrelated mechanisms. The association of Pgp expression in oral carcinomas wih resistance to anticancer agents has been demonstrated in few clinical studies. However, the unequivocal role of Pgp in clinical drug resistance in oral cancer remains to be ascertained. Several drugs that are currently being used for chemotherapy of oral cancer suchas cisplatin, methotrexate and 5-FU are not Pgp substrates. Therefore, lack of effective clinical response of some oral tumours to these drugs may be due to the involvement of non-Pgp mediated mechanisms of MDR. The clinical significance of non-Pgp mediated drug resistance mechanisms such as MRP1, GSTpi, p53, Bcl-xL, Ets-1, DNA topoisomerase II and DNA repair genes in oral cancer remains to be determined. Furthermore, different mechanisms of drug resistance may co-exist in the same cell and may be interconnected. For example, the inactivation of p53 (due to mutations or cytoplasmic sequestration by binding to cellular proteins) may result in two different types of MDR that appear to be connected: MDR connected with the activation of Pgp, as well as MDR connected with the inhibition of drug induced apoptosis. The MRP1 protein and the glutathione system may also be interconnected. Understanding the different defense systems of cells of the oral cavity which are activated on exposure of the oral mucosa to carcinogenic tobacco constituents and the signalling pathways participating in their regulation will provide further insight into multidrug resistance. These diverse mechanisms may ultimately merge into common cell death pathways which need to be elucidated.

The known candidate molecular markers for drug resistance and MDR modulators must undergo further analysis in large scale controlled prospective clinical settings involving examination of human oral tumour specimens and correlations with responses to chemotherapy. The multiplicity and diversity of MDR mechanisms hamper precise diagnosis of the causes of the patient's resistance to chemotherapy as well as the designing of strategies for MDR circumvention. In selecting the techniques for MDR studies, it is necessary to remember that several different mechanisms of drug resistance may be acting in the oral cancer cells, although there are mechanisms of resistance more often found after the influence of a particular drug on oral cancer cells. The resistance to cisplatin may be related to the activation of the glutathione system, increased drug efflux by MRP1, alterations in apoptosis regulation and increased DNA repair (Topoisomerase II).

Development and refinement of molecular methods for MDR detection and measurement in the clinical setting is of immense importance for predicting response to chemotherapy. Though different methods have been developed for the determination of the individual sensitivity of a patient's cells to drugs, presently the most popular technique entails uses of the primary cultures of a patient's cells for testing their sensitivity to the set of different drugs. The techniques used are: the tests of drug influence on cell proliferation; evaluation of the expression of the drug resistance genes and proteins (Pgp, Bcl-2 and GSTpi). A detailed understanding of MDR in oral cancer and its circumvention poses a major challenge for the future. Analysis of a tumour at the time of diagnosis to determine the expression levels of relevant drug resistance proteins (Pgp, MRP1, GSTpi, p53, Bcl xL, Ets-1, topoisomerases) may be useful to predict the response to chemotherapy. This knowledge may also be used to tailor an "individual" treatment protocol which should be aimed at circumvention of drug resistance upfront.

Clinical drug resistance in oral cancer may also be influenced by factors that affect drug delivery including tumour microenvironment.

host tumour interactions, inter cellular interactions, blood flow and permeability of tumour vasculature. In addition, nutrient deprivation and hypoxia within the tumour mass may promote drug resistance as these factors reduce the progress of cells through the cell cycle. These factors have not been discussed herein.

However, the heterogeneity of tumour cells and mutability will provide them with ways to manifest drug resistance. Major thrust has been laid on development of agents for reversal of Pgp mediated drug resistance. Future efforts should aim to prevent the emergence of drug resistance. This work is now being complemented by approaches that target cell death pathways such as those mediated by release of mitochondrial proteins and by activation of surface receptors such as Fas. Rapid progress has been made in developing small-molecular-weight drugs that influence the rate of apoptosis, for instance by binding to the bcl-2 family of proteins regulating mitochondrial permeability. Antisense approaches aimed at reducing bcl-2 expression, and thus increasing the rate of cell death, are also showing promise. New therapeutic approaches will have to be complemented by improved diagnostic tests to evaluate the contributions of different resistance mechanisms in individual patients with cancer. A better understanding of biochemical and molecular mechanisms of MDR in oral cancer may lead to development of novel strategies for more effective treatment of this malignancy. The successful management of oral cancer requires designing novel modalities including: biologic response modifiers, immune-based therapeutics and emerging pharmacologic agents triggering apoptosis in tumour cells.

DNA microarray analysis is likely to improve the ability to determine which drug resistance and drug metabolizing genes are upregulated in oral tumours and these results may be correlated with clinical responses to anticancer drugs to assess their clinical utility. DNA microarray analysis will unable us to determine which drug resistance and drug metabolizing genes are upregulated in different tumours. These results can also be correlated with clinical responses to chemotherapy. Using cDNA microarray Kudoh et al. (2000) compared the expression profiles of doxorubicin induced and doxorubicin resistant cancer cells. Transient doxorubicin treatment altered the expression of a diverse group of genes in a time-dependent manner. Subsets of the induced genes were also found to be constitutively overexpressed in cells selected for resistance to doxorubicin. This subset of overlapping genes may represent the signature profile of doxorubicin induced gene expression and resistance in cancer cells. Recent studies demonstrate that genomic approaches to chemosensitivity prediction are feasible. Staunton et al. (2001) developed an algorithm for classification of cell line chemosensitivity based on gene expression profiles. Oligonucleotide microarrays were used to determine the expression levels of 6,817 genes in a panel of 60 human cancer cell lines (the NCI-60) for which the chemosensitivity profiles of thousands of chemical compounds were known. The results of this study suggest that at least for a subset of compounds genomic approaches to chemosensitivity prediction are feasible. To explore genes that determine the sensitivity of cancer cells to anticancer drugs, Shingo etal. (2002) investigated using cDNA microarrays, the expression of 9216 genes in 39 human cancer cell lines pharmacologically characterized on treatment with various anticancer drugs. Some genes were found to be commonly correlated with various classes of anticancer drugs whereas other genes correlated only with specific drugs with similar mechanisms of action. This latter group of genes has been prposed to reflect the efficacy of each class of drugs. These reports suggest that the integrated database approach of gene expression and chemosensitivity profiles may be useful in the development of systems to predict drug efficacies of cancer cells by examining the expression levels of particular genes.

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