

WJCO 5th Anniversary Special Issues (1): Lung cancer**Apoptosis block as a barrier to effective therapy in non small cell lung cancer**

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the treatment of non-small cell lung cancers with platinum-based combination chemotherapy. New biomarkers of prognosis as well as new therapies focusing on molecular targets are emerging helping to identify patients who are likely to benefit from therapy. These are as yet only available to the minority of patients. Drug resistance remains the major cause for treatment failure. Apoptosis block as a mechanism for drug resistance and potential routes to overcome this will be reviewed.

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Abstract

Lung cancer, is the most common cause of cancer death in men and second only to breast cancer in women. Currently, the first line therapy of choice is platinum-based combination chemotherapy. A therapeutic plateau has been reached with the prognosis for patients with advanced non-small cell lung cancer (NSCLC) remaining poor. New biomarkers of prognosis as well as new therapies focusing on molecular targets are emerging helping to identify patients who are likely to benefit from therapy. Despite this, drug resistance remains the major cause for treatment failure. In this article we review the role of apoptosis in mediating drug resistance in NSCLC. Better understanding of this fundamental biological process may provide a rationale for overcoming the current therapeutic plateau.

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Key words: Apoptosis; Lung cancer; Adjuvant therapy; Mitochondria; BAX; BAK; BCL2

Core tip: A therapeutic plateau has been reached with

INTRODUCTION

Lung cancer is the leading cause of cancer death in the United Kingdom accounting for 24% of all male related cancer deaths and 20% in females^[1]. Similar trends are seen in the United States with lung cancer representing the most common cause of cancer death in men and women, recently overtaking breast cancer in the latter^[2]. In the United States, more than 213000 new cases were diagnosed in 2007 with a total of 1.61 million cases worldwide in 2008^[2,3]. The majority of these cancers (75%-80%) are non-small cell lung cancers (NSCLC)^[4].

Surgery is the mainstay of treatment for early stage and localised disease (Stage I and II and selected IIIA). Multimodal therapy is the norm for regionally advanced disease in the form of adjuvant chemotherapy. Palliative chemotherapy forms the mainstay of treatment in patients with advanced metastatic disease^[5]. Five year survival rates following lung resection for NSCLC are I A-73%, I B-54%, II A-48%, II B-38%, IIIA-25%^[6]. The majority of patients have advanced disease at the time of

diagnosis and therefore are not surgical candidates. This is illustrated by the fact that in the United Kingdom only 14% of patients diagnosed go on to have surgical resection^[7]. In those patients that are surgical candidates, more than 50% will develop a recurrence. Adjuvant chemotherapy has been used with limited success to decrease the recurrence rates but this has only yielded a survival benefit of 5%-15%^[8].

For patients not suitable for surgery due to advanced disease or those who have suffered recurrence following resection, chemotherapy forms the mainstay of treatment with evidence that platinum based therapies are most effective in the first line setting^[9,10]. Despite this, in a recent trial of platinum combination therapies in advanced NSCLC, only 30% of patients showed objective disease response and a significant proportion suffered toxic side-effects such as neutropenia (27%), anaemia (10%), thrombocytopenia (13%), alopecia (21%) and nausea (4%). During the trial, deaths due to study drug toxicity were in the region of 1%^[11]. This demonstrates the major problem of drug resistance in NSCLC to standard platinum based therapies and the associated toxicities.

MOLECULAR TARGETED THERAPY

A recent major advance in the management of NSCLC has been the identification of activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR)^[12]. These mutations, found in 10% of NSCLC specimens in the United States and Europe, are associated with increased sensitivity to EGFR tyrosine kinase inhibitors erlotinib and gefitinib^[13]. EGFR mutations are often accompanied by gene amplification^[14]. Mutation of the EGFR can lead to signal transduction independent of ligand stimulation, relaying signals to the PI3K-AKT-mTOR pathway involved in cell survival, or to the RAS-RAF-MEK-ERK pathway involved in cell proliferation^[15]. The fact that kinase inhibition leads to apoptosis in cells with mutant EGFR supports the notion that these cells are "addicted" to signalling *via* the mutant proteins^[16]. This explains the dramatic response to EGFR tyrosine kinase inhibitors in EGFR mutated NSCLC with associated improvement in survival^[17,18].

Molecular profiling in NSCLC has revealed a number of other mutations such as EML4-ALK translocation^[19]. This is associated with extreme preclinical sensitivity to respective ALK kinase inhibition in both preclinical and clinical settings^[20,21]. Additional molecular subclasses with associated somatic gene alterations have been discovered, predominantly in lung adenocarcinomas and include mutations of BRAF^[22], Her2^[23] and PIK3CA^[24]. The continual discovery of new molecular subclasses represents significant progress in the treatment of NSCLC but they should not necessarily be viewed as a panacea as resistance to these novel targeted therapies are being reported^[25]. The above molecular subclasses still only account for less than half all new cases of the disease. The majority of patients still rely on standard combination

chemotherapy of platinum based doublet regimens.

In addition to molecular tumour markers, there is increasing interest in developing biomarkers associated with NSCLC. These may be predictive for response to treatment or prognostic. An area of interest in our unit relates to the link between plasma fibrinogen and NSCLC. In early stage disease amenable to surgical resection, raised fibrinogen is associated with increased risk of incomplete resection and correlates with T stage of tumour^[26]. More recently, serum fibrinogen has been shown to be an independent predictor for both disease recurrence and overall survival in both resectable and advanced disease^[27,28]. The mechanism by which fibrinogen acts as a marker is not fully understood. Its role as an extracellular matrix, in promoting angiogenesis and metastasis has been proposed^[29-32].

APOPTOSIS AND DRUG RESISTANCE

Cisplatin (*cis*-diamminedichloroplatinum II) is the most effective platinum based therapy in the first line setting for NSCLC^[33]. It forms DNA-platinum covalent adducts resulting in inhibition of DNA replication, suppression of RNA transcription and protein translation, attempted DNA repair as well as disturbance of the cell cycle and the activation of apoptosis^[34].

Apoptosis serves as a natural barrier to cancer development^[35]. Normally the accumulation of genetic mutations required to drive uncontrolled cell cycle progression and tumorigenesis would result in triggering of apoptosis within a cell^[36]. The ability of cancer cells to evade apoptosis is, therefore, an essential hallmark of cancer^[37]. Defects in apoptosis, harboured by cancer cells, not only underpin tumorigenesis but also drug resistance^[38]. Resistance of NSCLC cells to a diverse range of cytotoxic therapies suggests a defect in apoptosis signalling^[39]. Further understanding of the mechanisms by which apoptosis resistance occurs and how this can be overcome will be important in order to administer a more rational approach to anticancer drug design and therapy.

APOPTOSIS

Apoptosis, a genetically encoded programme of cell death, was originally defined based on the morphological features observed as the cell died; nuclear condensation, nuclear and cellular fragmentation, membrane blebbing and phagocytosis of the dying cell in the absence of inflammation^[40]. The process of apoptosis is conserved in a wide range of multicellular organisms from worms to humans and plays a key role in normal development and homeostasis. The apoptosis phenotype is produced through the activity of cysteine-aspartic proteases, a family of cysteine proteases termed caspases. A hierarchical cascade of activation occurs which results in apoptosis. Two groups of caspases have been classified; the initiator caspases and the effector caspases.

The initiator caspases, caspase-8, -9 and -10, are ac-

tivated early in apoptosis signalling and have restricted cleavage targets, limited to self cleavage, the effector caspases and BID (caspase-8). In contrast, the effector caspases, caspase-3, -6 and -7, have hundreds of cleavage sites broadly distributed throughout the cell^[41]. The effector caspases are held in the cytosol as inactive dimers. The activating event, catalyzed by the initiator caspases, involves conversion to catalytically active enzymes by cleavage in the linker region between the large and small active subunits. This allows intramolecular rearrangements to form an enzymatically active dimer^[42]. Caspase-3 and -7 display highly similar substrate specificity and carry out redundant but essential functions in apoptotic cell death as mouse embryonic fibroblasts lacking both enzymes are resistant to intrinsic and extrinsic apoptotic stimuli^[43].

Caspase-8 and -10 are involved in the death receptor signalling pathway whereas caspase-9 is involved in mitochondrial apoptosis.

It is clear caspase activation is a critical step in the execution of apoptosis and is generally a terminal event for the cell. Regulation of the process occurs through the extrinsic, death receptor apoptosis pathway and the intrinsic mitochondrial apoptosis pathway.

EXTRINSIC APOPTOSIS PATHWAY

Apoptosis occurs via the extrinsic apoptosis pathway as a result of signalling through death receptors expressed on the surface of mammalian cells. In the 1970's it was identified that certain products of lymphocytes and macrophages caused the lysis of certain types of cells, especially tumour cells. This product was termed 'tumour necrosis factor (TNF)^[44,45]. It has been established that TNF has its effect via cell surface receptor binding and activation. At least 18 TNF family ligands and 29 receptors have been identified in humans^[46]. In cancer research, interest has grown around the use of TNF-related apoptosis-inducing ligand (TRAIL) and targeting its receptors, members of the TNF superfamily, since the observation that recombinant human (rh) TRAIL induces apoptosis in various tumour cells but not in normal cells^[47].

TRAIL activates apoptosis by binding to specific transmembrane receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5)^[48,49]. It can also bind truncated, non-functional receptors TRAIL-R3 and TRAIL-R4 known as decoy receptors (DcR1 and DcR2)^[50]. Upon binding TRAIL to the death receptors (DR4, DR5), trimerization of the receptors occurs and a complex is formed termed the death-inducing signalling complex (DISC). This is the key step for subsequent initiator caspase activation to occur.

INTRINSIC APOPTOSIS PATHWAY

The other major apoptosis pathway leading to caspase activation and cell death is the Intrinsic (mitochondrial) apoptosis pathway. As DISC formation is the key step

in extrinsic (death receptor) signalling so mitochondrial outer membrane permeabilization (MOMP) is the key requirement for caspase activation and apoptosis via the mitochondrial apoptosis pathway. As the name suggests, this apoptosis pathway is engaged primarily as a result of internal cellular stresses such as DNA damage, ER stress^[41].

MOMP during apoptosis is primarily controlled by the BCL2 family of proteins^[51]. The pro-apoptotic members, BAX^[52] and BAK^[53], contain BH1-3 domains. BAX and BAK are often referred to as effector proteins as there is an absolute requirement for their activation at the outer mitochondrial membrane for MOMP to occur^[54].

A diverse range of death signals caused by eg DNA damage, growth factor deprivation leads to a shift in the balance of anti- and pro-apoptotic BCL2 family members with signals produced to engage MOMP. Activation of a "multidomain" proapoptotic member, BAX or BAK, is an essential gateway to mitochondrial dysfunction required for cell death in response to these diverse stimuli^[54].

Upon receipt of a death signal, BAX translocates to the mitochondrial surface where BAK already resides^[55,56]. A detailed sequence of events from BAX translocation to the OMM, subsequent activation and MOMP has been described recently^[57]. The requirement for activated BAX and/or BAK for MOMP to occur is clear, the mechanism by which they bring about MOMP is not. The key feature of MOMP in bringing about caspase activation and apoptosis is cytochrome C release. This is clear from evidence that cells lacking cytochrome C fail to activate caspases in response to UV irradiation, serum withdrawal, or staurosporine^[58]. The primary function of cytochrome C is in oxidative phosphorylation as it is a key component of the electron transport chain and it is found loosely associated with the mitochondrial inner membrane. The other important mitochondrial intermembrane space protein released during MOMP is second mitochondria-derived activator of caspase (SMAC) also termed DIABLO (direct IAP binding protein with low pI). It binds XIAP's, antagonising their ability to inhibit caspases^[59,60].

APOPTOSIS BLOCK IN CANCER

Having outlined how apoptosis proceeds, the mechanisms by which cancer cells evade this process will be explored. Block in mitochondrial apoptosis has been broadly divided into three groups^[61]: (1) Class A block occurs when normal generation of proapoptotic activators by aberrant behavior is inhibited. The mechanisms by which aberrant behaviour, such as genomic instability and oncogene activation, generate death signals via BH3-only proteins is as yet poorly understood. This is an area of ongoing study; (2) Class B block occurs when there is a significant loss of the BCL2 family effectors, BAX and BAK; and (3) Class C block occurs when increased expression of an anti-apoptotic BCL2 family protein is present, thereby inhibiting or sequestering pro-apoptotic BH3-only proteins. In this scenario, the cell has generated

an appropriate BH3-only death signal but it is inhibited by opposing anti-apoptotic expression. Cells in this state are referred to as “primed for death” and are “addicted” to the overexpression of the anti-apoptotic protein^[62].

Apoptosis blocks such as described above may determine the sensitivity of a cancer cell to a wide range of diverse chemotherapy agents and explain frequently observed phenomenon of multidrug resistance^[51]. The fact that being primed for death (class C block) is apparently more common in tumours than in normal cells may help explain why chemotherapy is often more toxic to cancers^[51].

Having classified apoptosis block in tumours, a new technique for determining what type of block a given cell employs has been developed termed BH3 profiling^[61,62]. The basic method involves incubation of mitochondria isolated from tumour cells with a panel of BH3 peptides. By assessing the pattern of response, the type of apoptosis block present can be identified. This may be used in the future to select drugs targeting anti-apoptotic proteins as a strategy for improving efficacy of drug treatments.

EXPRESSION OF BCL2 FAMILY MEMBERS IN NSCLC

Much focus has been placed on the role of BCL2 as a prognostic predictor in NSCLC. Many studies over the last 15-20 years have assessed its expression in many differing solid tumours. Given its function as an antiapoptotic protein, it would be predicted that overexpression would result in a more aggressive and treatment resistant phenotype. The published data is mixed with regard to its role as a prognostic marker.

A meta-analysis from 2003 compiled 28 studies from 1993 to 1999 which report the expression of BCL2 in NSCLC and the prognostic value of its expression in the primary tumour^[63]. Immunohistochemistry was used to detect expression in all studies. Of the 28 studies included, 11 concluded Bcl-2 expression was a good prognostic marker, 14 concluded it was not prognostic for survival with only 3 linking Bcl-2 expression to poor prognosis. Having performed the meta-analysis, the authors concluded that Bcl-2 positive tumours had a significantly better survival than those with Bcl-2 negative tumours. Given its function in the apoptotic pathway this would appear a paradoxical conclusion. One theory suggests that loss of Bcl-2 expression correlates with tumour de-differentiation and therefore a more aggressive phenotype^[64]. As discussed above, increased expression of BCL2 would likely confer a class C block in apoptosis and as such these tumours would be primed for death perhaps explaining the increased survival. This should be interpreted with caution as a result of heterogenous treatment patients in these studies received. Given the highly complex interplay between Bcl-2 and its other family members in regulating apoptosis, it is not surprising that the study of one anti-apoptotic member yielded such a result. As knowledge about the interplay between Bcl-2

family members improves, other targets or combination of targets may be more relevant to study rather than a single protein in isolation.

The crucial role the proapoptotic multidomain proteins BAX and BAK play in mitochondrial outer membrane permeabilisation warrants further study as prognostic markers. Loss of both proteins would confer a class B apoptosis block. Many studies exist reporting the status of BAX and BAK expression in NSCLC but again only report in isolation and each is considered separately as prognostic markers.

Altered BAX expression is frequently reported in NSCLC^[65-68]. None of these studies conclude that altered BAX expression has significant value as a prognostic marker, although none have investigated the expression of BAK together with BAX. Fewer studies report the incidence of altered BAK expression^[69,70]. These studies report the incidence of BAK loss at 42% and 59%. They also include data on BAX expression with loss reported in 34% and 47%. Neither study reports the incidence of double loss. Both conclude that no prognostic value is attributed to BAX or BAK expression in NSCLC but again each protein is analysed in isolation. Data from the International Adjuvant Lung Cancer Trial (IALT) suggests a trend toward increasing chemosensitivity with increasing BAX level^[71]. The converse effect of BAX negativity was not reported.

STRATEGIES TO OVERCOME MITOCHONDRIAL APOPTOSIS BLOCK

Given the frequent loss of expression of each protein, it is likely a significant portion of patients with NSCLC will have BAX and BAK double loss and given the evidence that this results in a highly apoptosis resistant phenotype it would be important to assess the impact on double loss on both survival and response to standard chemotherapy and radiotherapy in NSCLC.

Given what is known about the mitochondrial apoptosis pathway, class B block in apoptosis is likely to prove resistant to a range of targeted therapies. BH3 mimetics would be predicted to be ineffective due to absence of effector proteins BAX and BAK. Alternative strategies will be required to treat these potentially multidrug resistant cancers. *In vitro* evidence exists to show that in the absence of BAX and BAK, detachment of hexokinase from mitochondrial VDAC can lead to cytochrome C release and therefore mediate cell death^[72]. This can be achieved using either a competitive peptide, or by inhibiting AKT (which in turn regulates hexokinase interaction with VDAC) and prove a rational approach to treating cancers exhibiting Akt activation or an imbalance in the expression of antiapoptotic and proapoptotic members of the Bcl-2 family.

CONCLUSION

NSCLC presents a major health burden worldwide. Plati-

num based chemotherapy is the mainstay of treatment in the clinic today, although denovo and acquired drug resistance has resulted in a therapeutic plateau since its introduction over 30 years ago. Novel targeted therapies are beginning to emerge that induce apoptosis in certain molecular subclasses. Apoptosis resistance underpins tumorigenesis and drug resistance. Understanding how apoptosis resistance occurs in NSCLC will allow tailoring of therapy and development of novel targets to overcome this problem.

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