Molecular Prognostic and Predictive Markers in Lung Cancer

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Abstract: Lung cancer is the most common type of cancer in the Western World and there has been systematic effort to find markers with prognostic and predictive significance. Molecular biology is lately being used in order to find markers that are highly sensitive, specific and associated with survival. This review presents recent data regarding the numerous studies that have been conducted in this field. No marker has yet been proven to offer significant prognostic and predictive information but there is need for further research with metanalyses and new prospective studies.

Keywords: Biomarkers, prognosis, pulmonary neoplasms.

INTRODUCTION

Lung cancer is the most common cancer in the Western World, with 1200000 new cases per year. Non-small cell lung cancer (NSCLC) constitutes the vast majority of cases as compared to small cell lung cancer (SCLC) : 85% versus 15%. The TNM staging system (Tumour, Node, Metastasis) seems to be the best prognostic system of overall survival in NSCLC patients [1]. Recent advances in molecular biology have led to a systematic effort to identify molecular markers which can help categorize patients in subgroups in order to achieve better therapeutic treatment, overall survival and quality of life.

CHARACTERISTICS OF A LUNG CANCER MOLECULAR MARKER

A tumour marker is defined as a protein or other substance that can be isolated in biological materials such as blood, plasma, sputum or tumour tissue of cancer patients. A tumour marker has prognostic value if it can predict outcome, regardless of the treatment administered. On the other hand, a marker is predictive, if it predicts the outcome of a certain treatment, therefore, it is useful for therapeutic decisions. We will review the literature on studies involving protein molecules which could be used as prognostic and predictive markers in lung cancer.

CELL CYCLE

There are two main stages of cell multiplication : the replication of all chromosomes during the interphase and the cell splitting into two distinct daughter cells during phase M of the cell cycle. The interphase consists of three distinct phases: G1 phase, S phase and G2 phase. Somatic cells have two check points between phases in order to control the process. The first checkpoint, the G1-S point, is the point where the somatic cell must respond to external signals and where it is decided whether the cell will duplicate or enter the G0 phase. The transitional points between phases are mostly controlled by cyclins and cyclin-dependent kinases (cdks) [2].

A. The following genes and their protein products play an important role in the G1-S phase and the first checkpoint:

1. Tumour Suppressor Genes

The Retinoblastoma (Rb) Tumour Suppressor Gene

Three genes that belong to this family have been isolated: pRb/p105, pRb/p107 and pRb2/p130. They seem to take part in the G1 check point of the cell cycle by inhibiting the transcription of certain genes whose protein products are necessary for DNA synthesis. The non-phosphorylated form of the retinoblastoma gene creats a complex with the E2F/DP1 key-transcriptional factor and hinders the transition from the G1 to the S phase of the cell cycle, therefore, blocks the cell duplication. Studies examining this gene's prognostic value have had contradictory results. Xu *et al.* [3] studied 101 NSCLC cases and found that the protein was expressed in only 24% of patients but these patients exhibited a 32-month overall survival as opposed to the 18-month overall survival in patients who did not exhibit Rb protein expression. Caputi *et al.* [4] came to the conclusion that when the protein is expressed in a lower degree or not expressed, it is correlated with poorer survival. In contrast, the biggest study conducted was not able to show that the Rb gene expression has prognostic value [5].

p53 Tumour Suppressor Gene

The p53 gene is named "guardian of the genome" because it inhibits DNA duplication when this is damaged. Under usual circumstances, the p53 oncogene activates the p21 tumour suppressor gene which hinders the transition from G1 to S phase of the cell cycle by creating protein complexes of the p21 oncoprotein with the D1/Cdk4 and E/Cdk2 cyclin/ cyclin-dependent kinase complexes. Furthermore, the p21 activation leads to the deactivation of the cyclin-dependent kinases Cdk4/6 and therefore, to the activation of the Rb tumour suppressor gene. Sanz-Ortega *et al.* [6] found that 22% of NSCLC smoker patients have LOH (loss of heterozygosity) at the TP53 locus, whereas this LOH is rare in patients without NSCLC. This offers the opportunity

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to detect smokers with mutated genotype in normal cells, and therefore, in greater risk of NSCLC. The study involving most patients didn't show a statistically significant difference in the survival of patients overexpressing the p53 protein [7] whereas smaller studies merely showed such a statistically significant difference [8]. A recent study based on 55 stage-I adenocarcinoma patients showed that p53 is an independent predictor of disease-free survival [9]. Studies that used molecular techniques to find mutations showed negative correlation of overall survival with the mutated p53 gene [10] with the exception of the Timotheadou *et al.* [11] study that found no prognostic significance. There was an effort to make sense of these contradictory results with three metanalyses. The Mitsudomi *et al.* metanalysis [12] concluded that the altered gene expression is a negative prognostic factor for overall survival of lung adenocarcinoma patients. Steels *et al.* [13] showed that the mutated p53 gene leads to poorer survival in adenocarcinoma as well as in squamous cell carcinoma in all stages of the disease. However, the Huncharek *et al.* [14] metanalysis showed that the p53 mutation can not be used as a prognostic factor in NSCLC. Yoshida *et al.* [15] showed that p53 can be a useful marker during follow-up of pure ground glass opacity on HRCT as its inactivation seems to be correlated to central consolidation within GGO of lung adenocarcinoma.

ARF Tumour Suppressor Gene

The INK4a oncogene, which encodes the ARF protein, is located on chromosome 9. The role of the protein is to inhibit the connection of the MDM2 protein to the p53 protein so that the cell is not divided. Numerous studies have shown that the ARF protein is the link binding the ras and myc oncogenes to the p53 tumour suppressor gene taking part in the mechanism of natural apoptosis. Inhibition of ARF expression through methylation or loss of the p53 gene or through inhibition of its expression are necessary steps of carcinogenesis. Recent studies have shown that alterations in ARF expression due to gene hypermethylation may have prognostic significance in certain subpopulations of NSCLC patients [16,17].

mdm2 Oncogene

This oncogene seems to take part in the cell cycle and in cell apoptosis, acting as an inhibitor of the p53 gene. It also contributes in the retinoblastoma oncogene expression pathway. Results concerning its use as a prognostic marker are contradictory. Studies have shown that the amplification of the oncogene can be associated with disease-free interval and overall survival, which are prolonged in patients without amplification [18]. However, Hu *et al.* [19] conducted a study based on patients of Chinese decent and came to the conclusion that there is no correlation between the various host genotypes and lung cancer.

FHIT (Fragile Histidine Triad) Tumour Suppressor Gene

This tumour suppressor gene also takes part in the apoptotic cell procedures *via* the p53-independent apoptosis pathway. Data to support its use as a prognostic marker do not suffice. Woenckhaus *et al.* [20] showed that it has prognostic significance in both squamous cell and adenocarcinoma histological types. However, in a study by Geradts *et al.* [21] there was no correlation between gene expression and overall survival.

2. Oncogenes

The Ras Oncogene

Three members of this family have been isolated in humans: H-ras, K-ras and N-ras. This oncogene takes part in the cyclin-dependent commencement of cell replication. Numerous studies have examined whether k-ras oncogene mutations can be used as NSCLC prognostic markers. Fukuyama *et al.* [22] showed that k-ras mutations are a negative prognostic marker for adenocarcinomas but not for squamous cell carcinomas. Rosell *et al.* [23] reached the conclusion that these mutations are a negative prognostic factor for disease relapse and morbidity for all stages and histological types. Other studies have shown no prognostic value of this molecular marker [24]. Because of these contradictory results, a metanalysis was recently conducted and results showed that k-ras mutations really constitute a prognostic marker for poor overall survival in NSCLC patients [25]. In the adjuvant trial JBR.10 it was found that patients with a RAS mutation did not benefit from adjuvant chemotherapy but this outcome was not proven statistically significant [26].

The myc Oncogene

It was shown that the C-myc oncogene amplification is related to lymph node metastases although it can not be associated with overall survival [27].

3. Growth Factors

Numerous growth factors and their receptors are expressed abnormally in lung cancer and constitute the initial stimulus for the cells to transcend from phase G0 to phase G1.

Epidermal Growth Factor Receptor (EGFR)

The c-erb-1 oncogene encodes the receptor of the epidermal growth factor (EGFR), which belongs to the family of the tyrosine kinase receptors. EGFR is a member of the family of ErbB receptors, along with HER 2, HER3 and HER4. Studies have shown that EGFR mutations are associated with certain patients' characteristics (women, non-smokers, adenocarcinoma histological type, Asian decent). EGFR mutations were shown to be prognostic for increased survival regardless of treatment and predictive of response to EGFR tyrosine-kinase inhibitors (EGFR TKIs), such as erlotinib and gefitinib. However, the TRIBUTE and INTACT trials showed that these mutations are not predictive of survival benefit owing to EGFR TKI treatment [28,29] and that is why there are ongoing studies examining the predictive value of EGFR gene amplification and EGFR protein expression for the differential effect of EGFR TKIs on survival. The BR21 trial has shown that high gene copy number is associated with 10 times greater response rate to erlotinib and, therefore, it is a better predictor of survival benefit from erlotinib as compared to EGFR mutations [30]. In the Hirsh *et al.* study [31], it was found that EGFR FISH-positive protein expression is a significant predictor of increased survival in patients receiving gefinitib. The Shepherd *et al.* [32] study was the basis for approval of erlotinib as a second- or third-line therapy for patients with metastatic NSCLC, as it was

shown that patients receiving erlotinib had prolonged survival.

Several studies have shown that EGFR and K-ras mutations are mutually exclusive and patients harboring K-ras mutations do not show benefit from EGFR-TKIs [28]. These findings suggest the presence of two different pathways of lung carcinogenesis, in which tobacco leads to K-ras mutations and exposure to a different carcinogen leads to EGFR-mutations.

Proto-Oncogene c-erb B-2

Even though it has been proven that this gene plays a significant role in breast carcinogenesis and its targeted therapy, its role in lung carcinogenesis is yet to be determined. Parra *et al.* [33] showed that higher c-erb B-2 expression was associated with greater metastatic capacity of the tumour. In the Cappuzzo *et al.* [34] study, it was found that patients positive for both HER2 and EGFR gene expression had better clinical outcome.

4. Cyclins and Cyclin-Dependent Kinases

Cyclins D and E are important in the cell cycle. Three different cyclin D types have been isolated (D1, D2, D3), which create complexes with the cdk4 and cdk6 cyclin-dependent kinases.

The Cyclin D - cdk4/6 Complex

It leads to the phosphorylation of the retinoblastoma tumour suppressor gene and therefore to cell replication. It was found that loss of D1 cyclins expression is associated with worse prognosis in all stages of squamous cell carcinoma [35]. However, Malusecka *et al.* [36] showed that, immunohistochemically, protein overexpression is not associated with histological type but with the stages.

The Cyclin E/cdk 2 Complex

This complex also leads to the beginning of the DNA replication. It has been proven that cyclins E are an independent prognostic factor and their overexpression is associated with poorer survival. Mishina al [37] found that cyclin E was ovexpressed in smokers, squamous cell carcinomas and advanced tumours and that protein hyperexpression is an independent prognostic factor. Another study showed that in adenocarcinomas, cyclin E expression was inversely proportional to tumour differentiation and protein overexpression was associated with poorer prognosis, whereas, in squamous cell carcinomas, protein expression was correlated to differentiation grade and better prognosis [38].

5. Cdk-Inhibitors

The initial theory was that their role in the cell cycle is to inhibite cyclin-dependent kinases, but they seem to take part in more complicated cell procedures. They are divided in two categories, the CIP/KIP family and the INK4 family.

The *CIP/KIP family* consists of the polypeptide inhibitors $p21^{cip1}$, $p27^{kip1}$, $p57^{kip2}$. The *p21* inhibitor blocks the continuation of the cell cycle by inhibiting the cyclin-D/CDK4 and the cyclin E/CDK2 complexes in the G1-S phase and the cyclin A/CDK2 in the S-G2 phase. Shoji *et al.* [39] showed that p21 expression is an independent prognostic marker. Komiya *et al.* [40] reached the same conclusion only for squamous cell carcinoma. However, the Dworakowska *et al.* [41] study had the opposite results. *p27* levels are raised when cells are not multiplying and its levels rapidly decrease during cell replication. Most studies show that the protein product of the p27 gene can be used as an independent prognostic marker in NSCLC patients of all stages [42] whereas the Esposito *et al.* [43] study did not show the same result. *p57* is a tumour suppressor gene which has an active role in many cancer types.

The *INK4 family* hinders the action of cyclin-dependent kinases D and therefore, the retinoblastoma tumour suppressor gene remains non-phosphorylated and the cell remains in G0 phase. The p16 inhibitor is the main member of the family. Studies have shown that it is associated with better survival. Kim *et al.* [44] studied the p16 and the FHIT genes and found that hypermethylation of both is correlated to greater relapse risk and shorter disease-free interval. Mohamed *et al.* [45] concluded that preoperative patients with pathologic N2 or even clinical N2 lymph node disease have better prognosis when they exhibit a positive p21 and p16 protein expression and are candidates for surgery.

6. Phase S of the Cell Cycle is Controlled by the Cyclin A/cdk2 Complex

Studies on cyclin A expression in NSCLC show that their overexpression is linked to poorer survival [46].

7. The Second Checkpoint G2-M is the Point Before the Cell Enters the Mitotic Procedure

The *cyclin B1/cdk2 complex* plays an important role here giving the signal for chromosome condensation, nuclear membrane destruction and mitotic spindle formation. Immunohistochemical studies have shown that cyclin B1 overexpression is associated with poorer prognosis [47].

CELL PROLIFERATION MARKERS

Ki-67: This is a nuclear antigen that can offer a quantitative approach of cell multiplication. Recently, a metanalysis including 37 studies was published and showed that Ki-67 overexpression is a negative prognostic marker in NSCLC [48].

Proliferating cell nuclear antigen (PCNA): PCNA is a nuclear protein which binds to DNA polymerase d. Published studies have presented contradictory results on the use of PCNA as a prognostic marker. A number of studies found positive correlation to phase S of the cell cycle and to overall survival, but Esposito *et al.* [49] were not able to prove an association of this nuclear antigen's overexpression with survival.

APOPTOSIS

Apoptosis is a normal cell process that leads the cell to its programmed death. The first apoptotic pathway is the death receptor pathway, which starts with the activation of initiator caspases by the cell surface receptors. The main caspases activators are Fas, Tumour Necrosis Factor Receptor-1 (TFNR-1) and Tumour Necrosis Factor Receptor-2 (TFNR-2). The second pathway is the mitochondrial pathway, consisting mainly of members of the Bcl-2 family, which includes preapoptotic factors (Bax, Bac, Bcl-xs, Bad, Bid) and antiapoptotic factors (Bcl-2, Bcl-X1, Bcl-w). The role of the

Bcl-2 gene was immunohistochemically studied in NSCLC patients of all stages without definite prognostic value being proven. The Yoo *et al.* study [50] showed that bcl-2 immunohistochemical expression was strongly correlated to better outcome and that it can be used as prognostic factor independent of the TNM stage in NSCLC. *Survivin* is an inhibitor of apoptosis and its increased expression is associated with poorer survival. A study on NSCLC patients that had undergone pulmonectomy or lobectomy showed that survivin expression in the cytoplasm is more frequent in squamous cell carcinoma but is not associated with survival, whereas survivin expression in the nucleus was proven to be an independent prognostic factor [51].

ANGIOGENESIS

It is generally considered that cancer cell proliferation, tumour growth and distant metastases are based on angiogenesis, which is the formation of new blood vessels and the reorganization of the pre-existing ones, so that the newly formed tumour continues to grow. Overall, angiogenesis is a complex procedure regulated by activating and inhibiting factors released by the tumour itself as by nearby tissues. The *Vascular Endothelial Growth Factor (VEGF)* is a growth factor for endothelial cells. Many studies have concluded that VEGF expression is inversely proportional to survival [46]. A metanalysis based on 20 studies was published in 2002 and it showed that VEGF expression is a negative prognostic factor in NSCLC [52]. Recently, the U.S. Food and Drug Administration (FDA) approved bevacizumab, an anti-VEGF factor, in combination with chemotherapy as first line therapy in non resectable NSCLC lung carcinoma [53]. Phase III trials are being conducted studying small-molecule inhibitors of the VEGF receptor tyrosine kinase, such as sunitinib and sorafenib, with promising results [54]. The *Basic Fibroblast Growth Factor (bFGF)* seems to play an important role in angiogenesis and tumour growth. Kojima *et al.* [55] studied the immunohistochemical expression in 132 stage I NSCLC patients and concluded that in squamous cell carcinoma, there is no relation to overall survival. The *Platelet-derived Growth Factor (PDGF)* takes part in DNA synthesis, endothelial cell migration and tumour growth. Results from studies on the PDGF prognostic value were non-conclusive. Koukourakis *et al.* [56] studied the immunohistochemical expression of PDGF in patients with resectable tumours but did not reach a definitive conclusion apart from the fact that the prognosis was worse in cases with PDGF expression even in abcense of lymph node metastases.

OTHER MOLECULAR MARKERS

Telomerase is an enzyme whose primary role is to elongate telomeres, which are shortened after every cell cycle, so that their length remains stable. In a large study based on 146 NSCLC patients of all stages, the hTERT (human telomerase reverse trancriptase) mRNA was studied and it was also shown that hTERT mRNA can be used as an independent survival prognostic marker [57]. On the contrary, no association of the sort was found in either the Hirashima *et al.* study [58] or in the Junker study [59].

GENES AND CHEMOTHERAPY

Two specific genes, the *ERCC1 (Excision Repair Cross-Completation group 1)* and the *RRM1 (Regulatory Subunit of Ribonucleotide Reductase 1)* have been associated with response to chemotherapy. It seems that the ERCC1 gene takes an active part in DNA repair after platinum-derived damage. It has been shown that ERCC1 expression is predictive of platinum-efficacy. It also appears to have prognostic significance but Rossell *et al.* [60] could not prove statistically significant impact on survival. The RRM1 gene takes part in nucleotide metabolism and influences the activity of antimetabolites, such as gemcitabine. Studies have shown that it has prognostic significance and predictive for benefit from gemcitabine chemotherapy [61].

CHROMOSOME ALTERATIONS

There are many chromosome alterations that happen in lung cancer constituting the basis of its pathogenesis. These chromosomal alterations are mainly chromosomal loss, loss of heterozygoty and gene hypermethylation and result in loss of tumour suppressor and of DNA repair genes, therefore in malignancy. The most commonly found alterations are related to loss of chromosome 3p and 9p regions or amplification of 1q and 3q region genes. Gene loss concerning the 3p region seem to be traced in 100% of lung cancer cells and in a large number of precancerous forms, leading researchers to the conclusion that it happens at an early stage of the lung carcinogenesis. In lung cancer, hypermethylation at promoter islands has been detected in numerous genes, such as CDKN2A, EX2, CDX2, and these could probably be used as markers in the future [62].

MOLECULAR PROGNOSIS AND STAGING IN SMALL CELL LUNG CANCER

The number of studies referring to SCLC molecular markers are far less that those in NSCLC.

Telomerase is overexpressed in most SCLC cases and this finding can be used in the development of targeted therapy in this disease [63]. Loss of the *FHIT* immunohistochemical expression has been associated to poorer survival [64].

The *p53* tumour suppressor gene has been extensively studied in SCLC but results do not prove that it can be used as reliable prognostic marker. A metanalysis on studies concerning p53 in SCLC was not able to show that it is a reliable prognostic factor [65]. The *c-erB-2 oncogene* was found to be overexpressed in 10% of newly diagnosed SCLC cases and it could be used as an independent prognostic marker in extensive disease [66]. The *myc oncogene* was studied in advanced disease stage and its overexpression may be related to worse prognosis. In the López-Martin study [67], c-kit protein expression had no significant impact on survival. *Growth factors* have been studied in peripheral blood and in the bone marrow of SCLC patients. In newly diagnosed SCLC patients, prepro-gastrin-releasing peptide (prepro GRP) and neuromedin B receptor (NMB-R) growth factor expression was increased only in patients with bone marrow infiltration and bone metastases. This overexpression has

been associated with poorer survival [68]. *Chromogranin A (CgA)* is a protein produced by neuro-endocrine tumours and seemed to correlate with disease stage and prognosis [69]. Studies have shown that angiogenesis is important in SCLC and is related to prognosis. VEGF levels have been measured in the serum of SCLC patients and when they are increased, the disease stage is advanced, response to chemotherapy is worse and overall survival is shorter [70]. On the opposite, a separate study on 54 patients did not show any relation between VEGF expression and overall survival [71].

CONCLUSION

Molecular biology is the leading force for achieving lung cancer molecular staging, prognosis and most of all, targeted therapy. The studies mentioned above concerning molecular markers are subject to a number of limitations owing to the different techniques used, the non homogenous populations studied and the declination in the number of patients in each study. Despite the intense research conducted, no highly sensitive and specific molecular marker has been isolated for lung cancer and the combination of multiple markers doesn't offer significant prognostic information. Furthermore, only few of these markers have undergone further research with metanalyses or prospective studies. Considerable progress has been made but important steps have yet to be taken.

ABBREVIATIONS

NSCLC	=	Non-small cell lung cancer
SCLC	=	Small cell lung cancer
TNM	=	Tumour-Node-Metastasis
Rb	=	Retinoblastoma
FHIT	=	Fragile histidine triad
EGFR	=	Epidermal growth factor receptor
EGFR TKIs	=	EGFR tyrosine-kinase inhibitors
cdk	=	Cyclin-dependent kinases
PCNA	=	Proliferating cell nuclear antigen
TFNR	=	Tumour Necrosis Factor Receptor
VEGF	=	Vascular Endothelial Growth Factor
bFGF	=	Basic Fibroblast Growth Factor
PDGF	=	Platelet-derived Growth Factor
hTERT	=	human telomerase reverse trancriptase
ERCC1	=	Excision Repair Cross-Completation group 1
RRM1	=	Regulatory Subunit of Ribonucleotide Reductase 1
prepro GRP	=	Prepro-gastrin-releasing peptide
NMB-R	=	Neuromedin B receptor
CgA	=	Chromogranin A

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