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REVIEW

Biomolecular and clinical practice in malignant pleural mesothelioma and lung cancer: what thoracic surgeons should know†

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Summary

Today, molecular-profile-directed therapy is a guiding principle of modern thoracic oncology. The knowledge of new biomolecular technology applied to the diagnosis, prognosis, and treatment of lung cancer and mesothelioma should be part of the 21st century thoracic surgeons' professional competence. The European Society of Thoracic Surgeons (ESTS) Biology Club aims at providing a comprehensive insight into the basic biology of the diseases we are treating. During the 2013 ESTS Annual Meeting, different experts of the field presented the current knowledge about diagnostic and prognostic biomarkers in malignant pleural mesothelioma including new perspectives as well as the role and potential application of microRNA and genomic sequencing for lung cancer, which are summarized in the present article.

Keywords: Malignant pleural mesothelioma • Lung cancer • Biomarker • microRNAs • Genomic sequencing

INTRODUCTION

Surgical practice is characterized by the continuous improvement of existing methods as well as the establishment of new techniques. To keep up with this ever changing and evolving field of our specialty, we are forced to constantly expand our knowledge. But studying takes time and is a difficult process to incorporate into the busy every day schedule with our commitment to patients' care.

So the aim of the Biology Club of the European Society of Thoracic Surgeons is to give a comprehensive insight into the basic biology of the diseases we are treating in addition to providing an overview of recent developments/techniques within the field. With a deeper understanding we might be able to bring the clinical problem from bedside to bench—a mission that cannot be accomplished by the pure scientist. This input is important for research, because the relevant problems are identified at the bedside. For many of these so-called translational projects between bench and bedside, tissue is the key factor, which is provided by the surgeon. The present article will provide an insight into various technologies available for the different analyses to be performed and is a summary of a compact session of the 'Biology Club' during the 2013 Annual Meeting of the Society in Birmingham.

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Although tumour molecular-profile-directed therapy is already guiding treatment for lung cancer patients, this is not presently the case for malignant pleural mesothelioma (MPM), but hopefully may be one day. In the first part of this article, perspectives for biomolecular diagnosis and prognosis in MPM are addressed and in the second part modern technologies that have brought new insights into the molecular basis of lung cancer will be summarized by experts in the field.

MESOTHELIOMA: NEW PERSPECTIVES FOR BIOMOLECULAR DIAGNOSIS

MPM has become an ever increasing model for biomarker discovery as evidenced by a doubling of yearly papers devoted to prognostic, diagnostic and predictive markers since 2000. It only makes sense to have this tumour as a research priority because of (i) the socioeconomic burden worldwide which is associated with asbestos exposure (AE), (ii) a ready-to-study high-risk population (AE) in many countries, (iii) the very short interval for maturation of intermediate end points for prognostic biomarker discovery and (iv) justification that there are long-term survivors with MPM and these long-term survivors are associated with early stage, epithelial histology, female sex and low volumes of disease when diagnosed. Armed with this information, experts in the MPM research field focus on developing minimally invasive, i.e. plasma-

or serum-based biomarkers, which can then detect the presence of a mesothelioma in the AE population who exhibits objective AE [1]. You cannot screen the over 4 million people in the USA who may have been exposed to asbestos; however, if there was a serum/plasma test that documented specifically revealed substantial AE was present, it would be in these individuals that longitudinal screening trials could be explored.

Jube *et al.* [2] have published that high-mobility group protein 1 (HMGB1), a damage-associated molecular pattern molecule that triggers the inflammatory response that characterizes programmed necrosis, is elevated in the serum of patients who have documented exposure to asbestos. These levels of HMGB1 are threefold higher than in individuals who were not exposed or who are present or former smokers. Levels of HMGB1, however, are also elevated in the serum of patients with MPM. Whether HMGB1 is an early marker of carcinogenesis in AE individuals remains a primary focus of the investigation of the Yang/Carbone Laboratory in Hawaii [2, 3], and in the near future a wealth of data defining the characteristics of those individuals which mitigate this rise in HMGB1, and a possible forthcoming topic of research is MPM prevention by the use of chemo-preventive agents against HMGB1.

Since HMGB1 is present in the serum of both AE and MPM, it will not serve as an early detection marker specific to MPM, since not all patients with elevated HMGB1 have MPM. Hence, the discovery and validation of novel serum- and plasma-based biomarkers has been a primary focus of the Early Detection Research Network (EDRN) Mesothelioma Biomarker Discovery Laboratory at New York University Langone Medical Center. After the groundbreaking work by Robinson *et al.* [4], describing the use of soluble mesothelioma-related protein (SMRP) for the diagnosis of MPM from AE in the Wittenoon Cohort of Western Australia, our laboratory along with Robinson performed a blinded SMRP validation of 817 AE vs 168 MPMs (manuscript in preparation, Harvey Pass). This trial, with the SMRP measured blindly at two separate laboratories, validated the area under (AUC) the received operating characteristic curve (ROC) of 0.80 not only in the entire cohort, but also in patients with Stage I or II disease. Subsequently, Hollevoet *et al.* [5] published an individual patient data meta-analysis of 1026 MPMs and 3465 controls based on data from 16 studies in the literature, and further confirmed an AUC of 0.80. SMRP, indeed, is a robust marker with good specificity, but its sensitivity has so far limited its application for early detection of MPM in high-risk longitudinally based studies. An EDRN sponsored assessment of the Beta-Carotene and Retinol Efficacy Trial (CARET) study in which 49 cases of MPM were diagnosed from 3897 AE individuals who contributed sera for the chemoprevention study revealed that the ROC of these 49 could generate an AUC of 0.72 1 year prior to the diagnosis using prediagnostic sera from these patients (manuscript in preparation, Harvey Pass). When the SMRP comparisons were performed any longer than 1 year prior to diagnosis, the ability to detect the disease was unsatisfactory.

The EDRN laboratory has used genomic technologies to define differences between matched pairs of mesothelium (normal peritoneum) and MPM from patients having cytoreduction for the disease. By using Affymetrix™ arrays with over 32 000 probes, a hierarchy of potentially robust extracellular secreted moieties has been studied and validated (or not validated!) using enzyme linked immunosorbent assays (ELISAs). A consistent but controversial molecule, osteopontin (OPN), has always shown significant fold increases in MPM compared with the control mesotheliums, with 9-fold elevations ($P < 2 \times 10^{-13}$). Indeed, the original manuscript described remarkable AUCs close to 0.90 for serum compared with AE and OPN increased in the AE population as a function of the appearance of the fibrosis, plaque or infiltrates on the computerized tomogram [1]. Unfortunately, it was learnt that OPN is not specific to MPM and also the inability to reproduce the results from the original paper was due to the fact that a thrombin cleavage molecule impacted on the levels of OPN measured when performed in serum [6–8] and that OPN ELISAs differed in reliability [9]. Subsequent investigators confirmed these findings by measuring OPN in plasma from MPMs and control populations with a rise in the AUC to levels comparable with SMRP [10–12].

Other markers including fibronectin and thrombospondin have been examined in the New York laboratory, but the most promising seems to be a member of the Fibulin family, Fibulin 3 (FBLN3), whose gene is epidermal growth factor receptor (EGF)-containing fibulin-like extracellular matrix protein 1 (EFEMP1) [13]. In the available literature, FBLN3 was found to be decreased in tumours compared with normal tissue while in MPM we found that it was 7-fold increased ($P = 10^{-9}$) compared with normal mesothelium. Our finding of upregulation was validated in silico by examining FBLN3 on expression arrays from Gordon *et al.* [14] and we then assembled cohorts of plasmas to examine FBLN3 using a commercially available ELISA including 142 MPMs, 136 AE, 43 healthy controls, 20 with ovarian cancer, 20 with breast cancer, 20 with glioblastoma and 31 with prostate cancer. Using MPM cohorts from Detroit and New York, the AUC for FBLN3 compared with any controls was consistently >0.95, and maintained 94% specificity at 100% sensitivity for Stage I or II lung cancers. Levels of FIBLN3 fell after successful cytoreduction and increased at the time of progression. A blinded validation using 48 MPMs and 96 AE from the Princess Margaret Cancer Center maintained an excellent AUC of 0.87. Moreover, pleural effusion FBLN3 was markedly elevated and specific to MPM effusions compared with benign effusions and effusions from other cancer histologies. Validation of these findings from other international sites is the next most pressing issue using either plasma in a prospective longitudinal trial or retrospective, longitudinally collected plasmas from high-risk AE.

Industry-related biomarkers for the diagnosis of MPM are also in validation trials, and the most promising of these is the SomaLogic 13 marker profile [15]. Using Somamers which are specifically designed short pieces of nucleic acids which bind selectively and specifically to individual proteins, a series of experiments comparing 79 MPMs with 80 AEs in a series of discovery and blinded validation sets led to the 13 marker algorithm which, like FBLN3, had consistently elevated AUCs above 0.95 in the discovery and validation sets. Moreover, in a blinded independent set of 38 MPMs and 62 AEs, an AUC of 0.95 was maintained. Individual SomaMer predicted proteins have been validated using commercially available ELISAs, and in a head-to-head comparison with SMRP with identical specimens, the Somalogic profile had much greater sensitivity and specificity. Further validation using serum from cohorts of other malignancies compared with MPM using the 13 marker panel is ongoing.

MESOTHELIOMA: NEW PERSPECTIVES FOR BIOMOLECULAR PROGNOSIS

Developing prognostic markers to decide whether to operate in the case of cancer is quite important because there is a desire to

help with aggressive therapy those patients who are likely to benefit from surgery and to avoid operating on patients who are unlikely to benefit. This is particularly true in the case of mesothelioma where surgery is associated with major morbidity and finite mortality, and even patients who recover well do experience pain and prolonged recovery. Multiple prognostic markers in addition to stage have been previously proposed for mesothelioma and include laboratory exams (platelet count, haemoglobin and white blood cell counts), tumour volume (as measured by computed tomography (CT)), histological subtype (epithelial vs non-epithelial) and lymph node status (as measured by position emission tomography-CT or preferably mediastinoscopy) [16]. Many of these markers can be obtained prior to definitive treatment and one should consider utilizing them in the decision-making process as to who is a reasonable surgical candidate. The Bueno lab has developed a molecular prognostic test based on gene expression. This prognostic test is determined by the geometric combination of three ratios of four genes [17]. It has been developed using microarray analysis and has been validated in 3 retrospective cohorts as well as in 1 prospective cohort of 160 patients who underwent extrapleural pneumonectomy or pleurectomy/decortication. This test remains significant in a multivariate model which includes all the other known prognostic tests, leading the Bueno lab to propose a prognostic score that combines the molecular test with tumour volume, histological subtype and lymph node status, an approach that has recently been validated in an independent specimen cohort. While this test has been developed in frozen tumour tissue, it has recently been converted successfully to a formaldehyde fixed-paraffin embedded tissue-based test. This test has been commercialized and should be available by early 2014.

Experience with other prognostic tests indicates that the addition of tests from orthogonal platforms can add accuracy. With the advent of new genomic platforms and technologies, it is expected that additional such tests may be added to the prognostic test to increase its accuracy. However, as additional groups explore new tests, it is important to point out that rigorous validation in sufficiently robust independent cohorts including all currently known prognostic variables be carried out prior to adding these tests to the clinical armamentarium.

LUNG CANCER: THE ROLE OF MICRORNAS

MicroRNAs (miRNAs) are 19–25-nucleotide-long non-coding RNAs that regulate gene expression by binding complementary sequences of target mRNAs and inducing their degradation or translational repression. Strongly conserved among distantly related organisms, miRNAs are involved in a variety of biological processes including cell cycle regulation, differentiation, development, metabolism, neuronal patterning and aging [18]. Alterations in miRNA expression are involved in the initiation, progression and metastasis of several human tumours, including lung cancers [19].

Many studies have demonstrated the critical role of miRNAs in lung cancer pathogenesis and their potential as biomarkers for lung cancer risk stratification, outcome prediction and classification of histological subtypes. In 2004, the first report was published showing the potential clinical and biological effects of such an miRNA alteration in lung cancer. The authors found that let-7 expression, which regulates kirsten rat sarcoma viral oncogene homolog, was reduced in lung cancer tissues and was also associated with shorter survival after surgical resection [20]. Reintroduction of let-7 has been shown to functionally inhibit non-small-cell lung tumour development in mouse models [21].

In addition to lung cancer epithelial tissue, stromal components could also be an appealing source of early biomarkers, since they are likely to be affected by carcinogen exposure and strong inflammatory conditions such as by chronic obstructive disease. These are for aggressive lung cancer. Recently, a strong prognostic gene-expression signature, validated in independent datasets, was derived from cancer-associated fibroblasts isolated from 15 non-small-cell lung carcinoma samples compared with matched normal fibroblasts [22]. Another group showed that miRNA expression profiles associated with aggressiveness of the disease and poor survival were found not only in lung tumours, but also in adjacent normal lung tissue of the tested patients [23]. All these findings strengthen the hypothesis of the existence of a lung microenvironment, likely smoke-related, conducive to tumour growth.

miRNAs derived from the cancerous tissue or from the tumour microenvironment could also constitute a new class of blood-based biomarkers useful for cancer detection and prognosis definition, since, for their nature, they seem to remain rather intact and stable [24]. Development of non-invasive blood-based biomarkers for cancer detection in its preclinical phases is crucial to improve the outcome of deadly diseases such as lung cancer. Initial studies in lung cancer patients showed that plasma or serum levels of specific miRNAs had remarkable sensitivity and specificity to distinguish cancer from healthy subjects.

Reasoning that ideal miRNA biomarkers should be identified before the onset of the tumours and be able to predict aggressive vs indolent disease development, it has been found that ratios among 24 miRNAs were able to discriminate samples collected at disease or up to 2 years before the disease detection from individuals enrolled in two independent low-dose computed tomography (LDCT) screening trials. Moreover, specific signatures could identify correctly subjects who will develop aggressive lung cancer [23]. In particular, some miRNAs were more important in predisease than in diagnostic signatures and *vice versa*. This might be explained by the consideration that genes and pathways necessary in the earlier phases of disease development are different from those required for tumour maintenance and progression. Analysing the performance of these signatures in samples collected predisease, at the time of disease detection and post-surgery, the reproducibility of plasma miRNA test between the predisease and disease samples was observed (manuscript in preparation, Ugo Pastorino). In addition, positivity to miRNA signatures fell to negative after surgery in the majority of subjects, thus confirming the specificity of the plasma miRNA assay.

These preliminary data have been recently validated in combination with LDCT in a larger series composed of more than 1000 individuals enrolled in the Multicentre Italian Lung Detection trial [25]. Although LDCT is currently the standard of care for early lung cancer detection [26], it results in a general over diagnosis of indolent nodules, thus increasing individual radiation exposure, harmful confirmatory diagnostic procedures, unnecessary surgery, overload of highly specialized medical centres and increased costs for the healthcare system [27]. Non-invasive circulating miRNA assays could overcome most of these problems by exploiting the synergy between the molecular and imaging tests to reduce the number of the false positives.

LUNG CANCER: POTENTIAL CLINICAL APPLICATIONS OF GENOMIC SEQUENCING

There have been considerable advances since the discovery of the human genome in 2004 [28] and while 'genomics' is sometimes used as a loose term for cancer genetics in general, it applies to methods that involve DNA sequencing. There have been many advances in cell biology and genetics that are relevant to the thoracic surgeons including disorders of the genome (EGFR receptor mutations), the transcriptome (mRNA and miRNA diagnostic and prognostic profiling), the proteome (melanoma antigen family A, 3 expression for immunotherapeutics) and in future the metabolome (products of cell metabolism). In this section, we focus on gene sequencing of DNA.

DNA sequencing technology continues to improve, with an exponential increase in output per instrument run between 2004 and 2006 [29], and the costs and time required continues to drop significantly [30]. Currently, there are different scales to which the genome can be sequenced, at the level of the whole genome, whole exome and targeted sequencing. Whole genome sequencing includes both intron (coding regions of the DNA) and exons (non-coding regions of the DNA). This is the complete DNA sequence and the information yield is often unwieldy requiring specialized bioinformatics input for interpretation. This can now be achieved with Next-Generation Sequencers; however, the output is so large that it is difficult to interpret and suitable only for research at this stage. The next level is whole exome sequencing, which reveals the coding region of the DNA and currently a lot of biology and exploratory work is being undertaken at this level to help understand carcinogenesis, cancer biology and the search for new drug targets [31].

Currently, the level that is most applicable is targeted sequencing, which covers only the DNA regions of interest. This can be achieved by a number of different techniques ranging from Next-Generation Sequencing to simple polymerase chain reaction. In clinical practice, these techniques have been used in research on diagnosis, prognosis and treatment predictions.

Plasma quantification of DNA in lung cancer has been undertaken by Sozzi *et al*. [32] in an attempt to develop a blood-based marker for lung cancer diagnosis with promising results in the development cohort with an ROC_{AUC} of 0.94 (0.91–0.97); however, the level of discrimination dropped in a further validation cohort to conclude that plasma DNA was not different in individuals who developed CT-detected lung cancers vs cancer-free control subjects and was only slightly higher at the time of cancer diagnosis [33].

There is no doubt that among the most important discoveries in today's management of lung cancer is the presence of drug treatable mutations (EGFR and anaplastic lymphoma kinase) leading to considerable improvement in lung cancer survival [34–36]. In the surgical setting, randomized double-blind trial in adjuvant NSCLC with tarceva is a study of adjuvant erotinib in post-surgical patients with EGFR drug sensitizing mutations and we are currently awaiting the results. Perhaps one of the most interesting and relevant uses of DNA mutation testing in surgery is the setting of preoperative induction treatment. Researchers in Toronto recently published an abstract on the outcomes of preoperative erlotinib in 22 patients reporting evidence of radiographic regression of tumours [37] opening the door for the consideration of targeted induction treatment.

Other recent advances include blood-based DNA mutation testing, and we and others have published on the ability to capture circulating tumour cells [38] with the potential of DNA extraction and mutation testing.

In summary, genome sequencing can now be undertaken with greater output, higher speed and lower costs. While whole genome and exome sequencing are currently research techniques, targeted sequencing is already being applied in clinical practice. Improvements to genome sequencing may guide research surgical practice through adjuvant treatment, induction treatments and future minimally invasive methods of lung cancer diagnosis. All this emphasizes the importance of surgeon participation in tissue banking and basic translational research.

CONCLUSION

The appreciation of concepts such as multiclonality, genomic signatures and epigenetics represents the foundation of translational medicine in thoracic oncology [39–42]. The clinical implications of this triad are potentially manifold and only partially understood at this point in time [43, 44]. In this setting, thoracic surgeons are not simply spectators of this new era but rather main actors with their clinical acumen, surgical skills and central role in the diagnostic and therapeutic pathways of lung cancer and mesothelioma [40, 45, 46]. The ever evolving knowledge of the most frequent genetic mutations and their significance either as predictors of drug response or as prognosticators represents a fundamental part of the modern thoracic surgical practice [40, 46–48]. Moreover, new perspectives are disclosing that will require further reshaping the professional profile of thoracic surgeons who will increasingly rely on additional diagnostic capabilities in the selection of surgical candidates [49, 50]. Indeed, multidisciplinary involvement in thoracic oncology cannot thrive without this knowledge, which is an essential 'Esperanto' of lung cancer and mesothelioma tumour boards in an effort to improve the quality of service to our patients [51].

Conflict of interest: Raphael Bueno received a grant support from Castle Biosciences from 2012–2013. Harvey Pass declares that he has patents for the use of mir-29c*, fibulin 3 and osteopontin as biomarkers for diagnosis and prognosis of mesothelioma. Eric Lim performes consultancy or is in the advisory board of Strategen, Abbott Molecular, Glaxo Smith Klein and Pfizer and received speaker fees from Roche, Imedex, Glaxo Smith Klein and Boehringer Ingelheim. He received travel, accommodation and course fees from Covidien and research funding from ScreenCell (R). He also declares a patent (ClearBridge BioMedics). The remaining authors have nothing to declare.

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