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Molecular classification of tumors with special reference to *EGFR* mutation in lung cancer

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Abstract Unsupervised hierarchical clustering in expression profiling analyses allows molecular classification of tumors based on the similarity of genome-wide expression patterns. This new molecular-based classification shares the current pathological classification in part, although it also provides additional clues to identify cancers by their biological groups. Herein, we introduce a novel means of molecular classification of lung cancer. When examining the gene expression profiling analyses of lung cancers published so far, the molecular classification differs from the current classification schema, dividing lung cancers into two distinct branches that do not segregate SCLC and NSCLC, as might be expected. One of the branches includes adenocarcinoma alone, which is associated with a normal expression profile, while the other branch includes all four histological subtypes. We further examined the adenocarcinoma subset of the first branch. This subset of adenocarcinomas is characterized by frequent development in females and non-smokers, expression of thyroid transcription factor-1 and surfactant proteins, and specific involvement of epidermal growth factor receptor (*EGFR*) gene mutation. Furthermore, this subset is highly distinctive not only among lung cancers but also among carcinoma arising in other tissue sites, in terms of *EGFR* gene mutation and expression profiles. Although further studies are needed to clarify this adenocarcinoma subset, the distinction should be taken into consideration in lung cancer research and clinical strategies for treatment.

Keywords *EGFR* · Molecular classification · Expression profiling analysis · Lung cancer · Bronchioloalveolar carcinoma

Introduction

The completion of the human genome project now makes it possible to perform genome-wide analyses. Expression profiling analysis is such an example, and by comparing genome-wide expression patterns we can classify tumors according to their biological characteristics (Fig. 1). Unsupervised hierarchical clustering is commonly used for this purpose, and therefore the clustering represents the molecular classification of tumors.

Using this clustering method, breast cancers have been analyzed [21]. Breast cancers have been molecularly classified into four distinct subtypes including normal breast-like, luminal cell-like, basal cell-like, and HER2 subtypes [21, 26–28]. This molecular classification schema has been reconstructed with various series, and specific involvement of *BRCA1* mutation in basal cell-like types supports the biological significance of the classification [6, 27]. Genome-wide status of loss of heterozygosity has also revealed that breast cancers can be divided into four subtypes that are identical to the molecular classification using expression profiling

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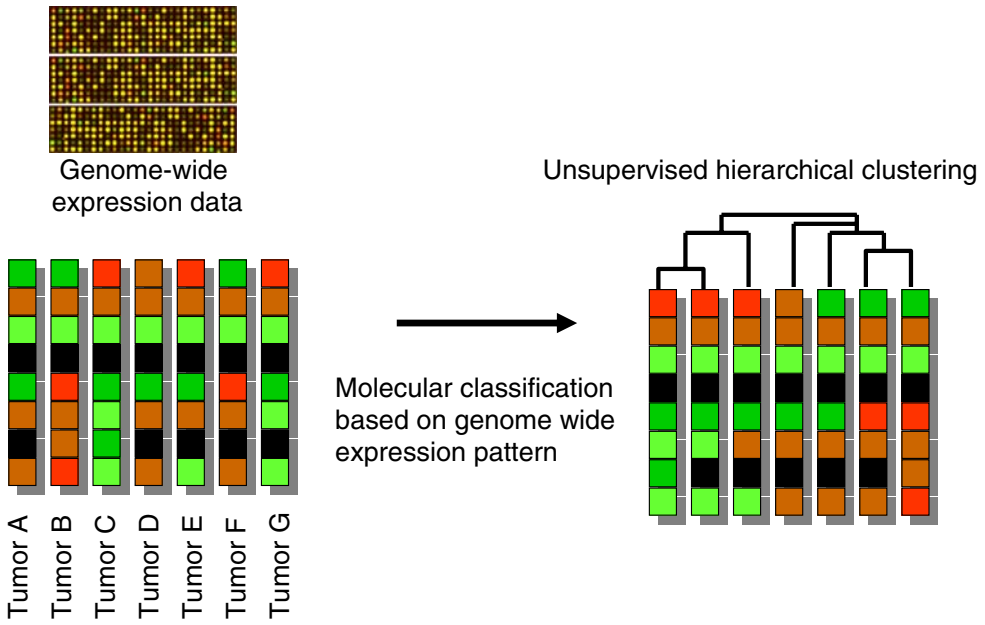

Fig. 1 Molecular classification based on genome-wide expression pattern. By comparing their genome-wide expression patterns tumors can be classified according to molecular–biological characteristics. Unsupervised hierarchical clustering is commonly used for this purpose, and therefore the clustering represents the molecular classification of tumors

analyses [32]. Recently, it has been reported that the breast cancer subtypes defined molecularly are associated with the clinical response to neoadjuvant chemotherapy [23].

In expression profiling analysis of lung cancers, according to data published so far, four major histological subtypes—adenocarcinoma, large cell carcinoma, small cell carcinoma, and squamous cell carcinoma—are recognized as distinct clusters [2, 3, 8, 10, 31]. However, the molecular classification distinctly differs from that used in current therapeutic strategies, in that molecular classification highlights the high diversity of lung adenocarcinomas and places SCLC and NSCLC in close relation. In this article, we examine and attempt to interpret the biological significance of this diversity.

Diversity of lung adenocarcinomas

Based on the available data, the molecular classification schema for lung cancers is summarized in Fig. 2, although the proportion of each cluster varies among reports [2, 3, 8, 10, 31]. In general, lung cancers branch off into two groups then cluster into histological subtypes. The left branch of the schema includes adenocarcinoma alone, which is associated with the profile of normal lung tissue, while the group in the right branch is composed of all four histological subtypes. Namely, lung adenocarcinoma can be divided into two subsets of right- and left-branched groups. The left branch corresponds to "adeno-group 1" and "adeno-group 2" in the study by Garber et al. [8], C3 and C4 according to Bhattacharjee et al. [2], group 2 in Borczuk's work [3], and AD3 and AD4 in our study [31]. All these studies describe this subset of adenocarcinoma as being characterized by molecules expressed in the peripheral lung parenchyma such as thyroid transcription factor (TTF)-1 and various isoforms of surfactant proteins that suggest a close association with peripheral lung parenchyma. They also support the idea that this subtype is always clustered together with normal lung tissues.

In contrast, the adenocarcinoma group of the right branch is mixed with the clusters of squamous cell carcinoma and SCLC. Notably, Bhattacharjee et al. [2] include in their analysis the expression profiles of metastatic lung cancers from the colon. Surprisingly, this metastatic carcinoma is stationed within this right branch and adjacent to lung squamous cell carcinoma, but not as a single cluster isolated from the lung cancers, implying that genome-wide expression patterns of metastatic adenocarcinoma are rather similar to those of lung squamous cell carcinomas, and that the differences between metastatic carcinoma and lung squamous cell carcinoma are less pronounced than those between right and left groups of lung adenocarcinomas.

When adenocarcinomas arising from various organ sites were compared, lung adenocarcinoma was distinguished by expression of certain characteristic

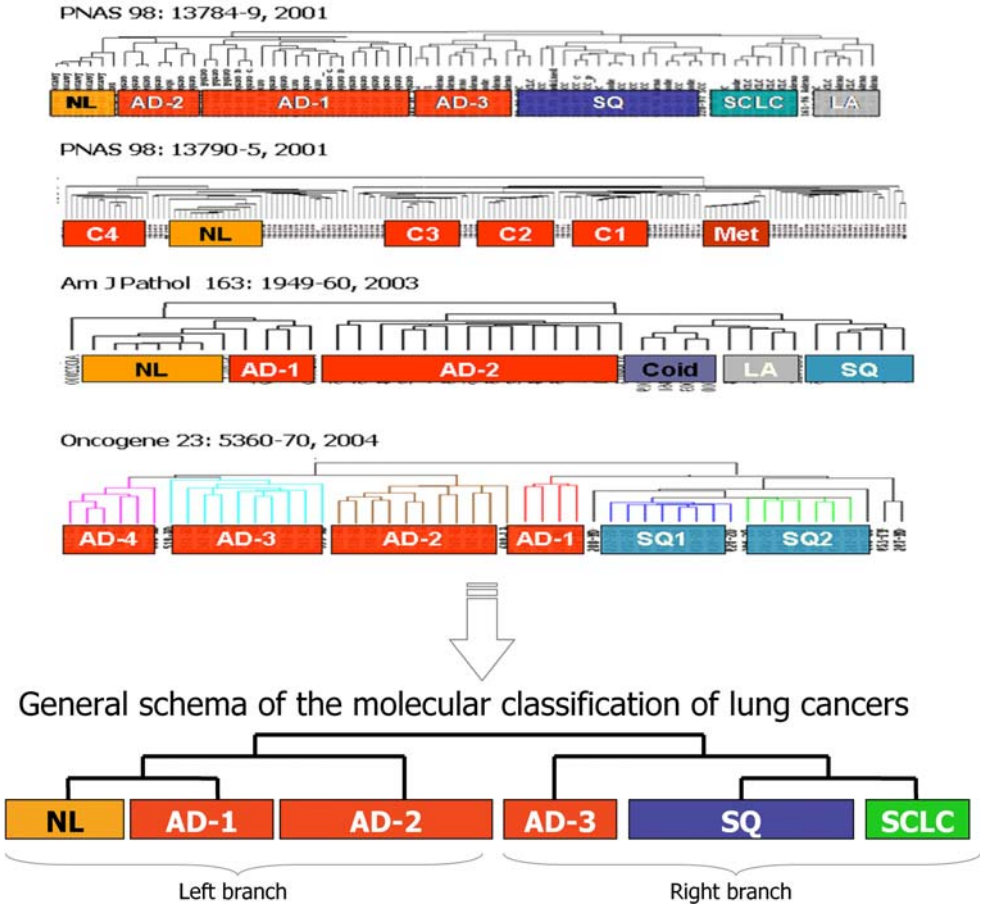

Fig. 2 Molecular classification of lung cancers. Four representative molecular classifications, modified in order by the author, reveal almost identical features (summarized in lower panel). Lung cancers branch off into two groups then cluster into histological subtypes

molecules [9, 24]. These always included markers of peripheral lung parenchyma such as TTF-1 and surfactant proteins, which are also selected as markers of the left branch. These findings indicate that the adenocarcinoma subset of the left branch is highly distinctive not only among lung cancers but also among adenocarcinomas arising from various tissue sites. This relationship may be analogous to that of whales, dolphins, and fish living in the sea (Fig. 3). Although all these creatures live together, whales and dolphins are mammals, not fish. In comparison with the distinct subset of the left branch, the difference between lung squamous cell carcinoma and metastatic cancer may be very small.

Terminal respiratory unit and its adenocarcinomas

Anatomically, normal lung tissue is composed of an air-conducting system and peripheral lung parenchyma wherein gas is exchanged. Bronchial surface epithelium, bronchial glands, bronchial cartilage, and muscles make up the former system, while pneumocytes, unique cells responsible for gaseous exchange, and capillary vessels are the main components of the peripheral parenchyma (Fig. 4). Two phases are observed in fetal/neonatal ontogeny of the lung [4, 16]. Branching morphogenesis from lung buds is initiated, and the conducting system is generated. Development and maturation of the parenchyma follow, and alveolar differentiation is completed in the first few weeks after birth. The adenocarcinoma subset seen in the left branch of the molecular classification appears to be derived from epithelium of the peripheral parenchyma.

Each adenocarcinoma subtype in the molecular classification is characterized by its expression pattern of certain molecules [2, 3, 8, 10, 31]. Interestingly, characteristic markers for the left branch subset are almost identical. Among them, we have focused on TTF-1. This molecule is a transcription factor that regulates functionally important molecules for respiration (e.g., surfactant proteins) [5, 19, 33]; TTF-1 knockout mice specifically lack peripheral lung parenchyma but not bronchial tree [11, 18]; and TTF-1 exhibits restricted expression in human pneumocytes and non-ciliated bronchial epithelium from early development to adult lung [20, 29, 36]. These findings suggest that TTF-1 functions as a master key regulator of the peripheral lung parenchyma. Indeed, when examined in a normal

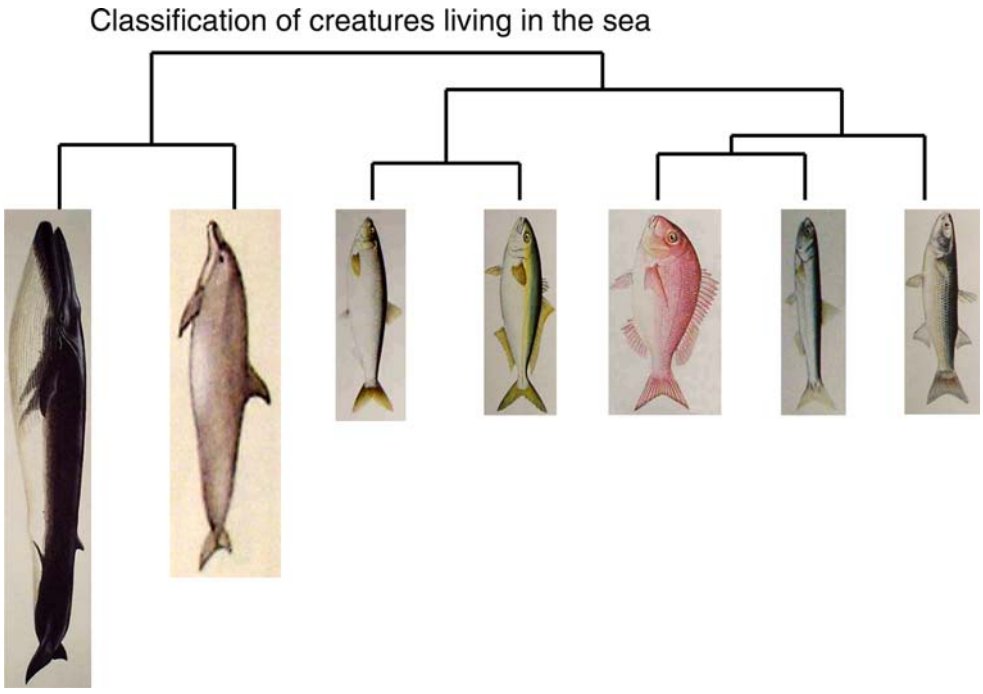

Fig. 3 Example classification of sea creatures showing analogy to molecular classification of lung cancers

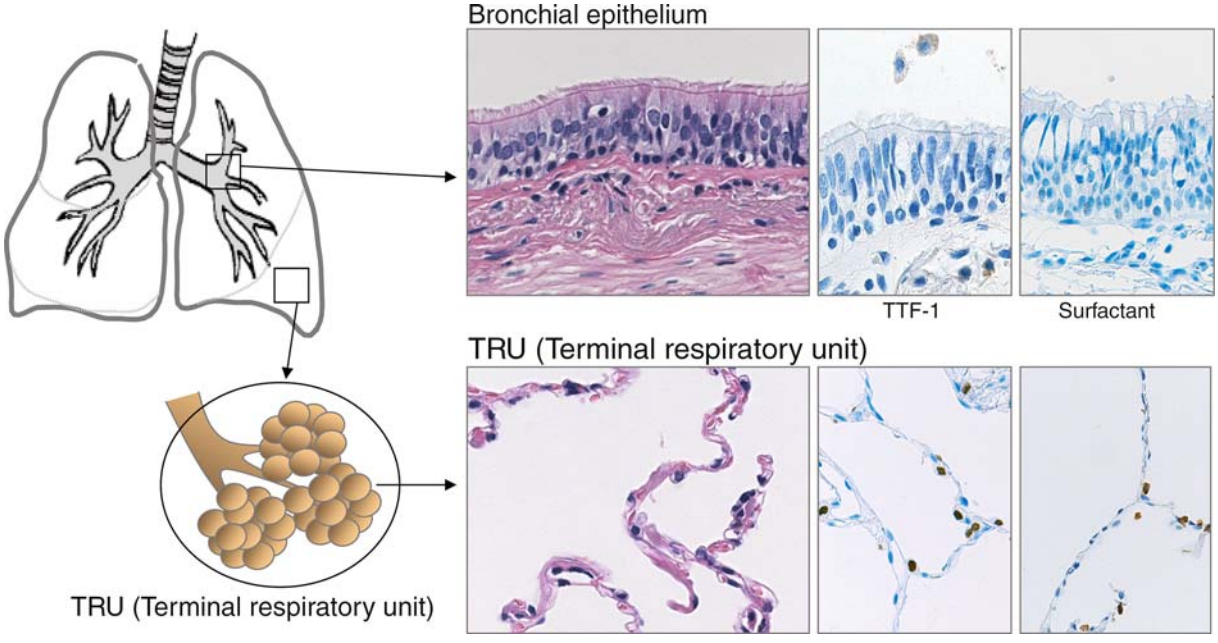

Fig. 4 The two major components of the lung: an air-conducting system and the terminal respiratory unit (*TRU*). TRU shares expression of the left branch of adenocarcinomas in the molecular classification

lung the expression is so uniform that it appears to represent a functional unit or cellular lineage. We therefore term this the "terminal respiratory unit (TRU)" [34–36] (Fig. 4).

We examined the clinicopathological characteristics of TRU-type adenocarcinoma using TTF-1 as a marker for the TRU feature. TRU-type adenocarcinoma is frequently observed in females and non-smokers [36]. Histologically, the cellular features of TTF-1-positive adenocarcinoma resemble TRU cells, whereas TTF-1-negative adenocarcinomas appear derived from bronchial glands and/or bronchial surface epithelium. Furthermore, cancer-associated genes including *p53*, *KRAS*, and *p27* show different expression patterns between TTF-1-positive and -negative adenocarcinomas, suggesting different molecular pathogenic mechanisms [34, 36].

In 2003, two phase II trials of gefitinib, Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL) 1 and 2, demonstrated that certain subsets of patients (namely Japanese, females, and those with adenocarcinoma) appeared to have higher response rates [7, 13]. Subsequent analysis by Miller et al. [17] revealed that smoking history and bronchioloalveolar pathologic subtypes were predictive factors for positive response to gefitinib. These features overlapped with those of TRU-type adenocarcinoma, suggesting some association. Therefore we examined the correlation between epidermal growth factor receptor (*EGFR*) mutation and TRU-type adenocarcinoma [12]. In a total of 241 consecutive NSCLC samples, *EGFR* mutation was detected in 97 adenocarcinomas and one adenosquamous carcinoma. More than 90% of the *EGFR*-mutated tumors expressed TRU markers including TTF-1 and surfactant proteins and showed morphological features of TRU cells. This specific involvement of *EGFR* mutation in TRU-type adenocarcinoma was supported by multivariate analysis. A logistic regression model revealed that only smoking status and cellular lineage (TRU vs. non-TRU type) were significant factors affecting *EGFR* mutation. Therefore it was concluded that EGFR mutation is specifically involved in TRU-type adenocarcinoma.

Interpretation and biological significance of the molecular classification

Gene mutation in the EGFR kinase domain is extremely rare in extra-pulmonary cancers; it was only detected in a case of colon cancer through the examination of >1,000 cancers arising from organs other than the lung (Table 1) [1, 14, 15, 25]. Although a large number of hematological cancers and sarcomas harbor characteristic gene alterations, such tumor-specific gene alterations are very exceptional in epithelia-derived cancers. However, this enigma may be explained by the characteristics of TRU cells and the specific involvement of *EGFR* mutation in TRU-type adenocarcinoma. As mentioned above, TRU cells are unique, being the only cells involved in gaseous exchange. Therefore only the lungs contain TRU-like cells. *EGFR* mutation is specifically involved in TRU-type adenocarcinoma, suggesting that TRU cells may have a specific susceptibility to acquisition of *EGFR* mutations. This explanation is consistent with the molecular classification findings.

Separation of these two distinct groups of lung cancers may also provide important insights for lung cancer research. The molecular difference between the two groups is particularly distinct, while *EGFR* mutation is specific in the left TRU-type branch of the molecular classification. Therefore when the molecules differentially expressed in lung cancers with and without *EGFR* mutation are compared, it is likely that the result represents only the difference between the two distinct groups and not upregulated or downregulated molecules derived from *EGFR* gene mutation. Indeed, our recent article [30] reported that three of the top six genes differentially expressed in tumors with and without *EGFR* mutation are identical to those with TRU and non-TRU features. This indicates that selection of proper objective indicators is important to obtain biological significance in cancer research and clinical investigation. A recent article on breast cancers [22] exemplifies this significance: X-chromosome abnormalities were specifically found in basal-like breast cancers, and therefore it was suggested that this alteration plays an important role only in this subset. This abnormality might otherwise have been mistaken for a rare alteration if this subset was not considered.

Consideration of this remarkable distinction may help us develop better therapeutic strategies for lung cancers. It is quite reasonable that metastatic adenocarcinoma from the colon is treated differently from lung adenocarcinoma, because the tumors are of different organs. However, TRU and non-TRU-type adenocarcinomas are currently treated under the same therapeutic strategy, although differences between the expression profiles of these tumor types are much greater than those between metastatic cancer of the colon and squamous cell carcinoma of the lung. As in the case of breast cancers [23], it may be interesting to examine the therapeutic response by molecular subtypes using specimens under clinical trial conditions.

Table 1 Gene mutation of EGFR kinase domain in extra-pulmonary cancers

Tumor(s) examined	References				Total
	[15]	[25]	[14]	[1]	
Breast	0/141	0/31	0/93	–	0/265
Head and neck	0/65	–	–	–	0/65
Pancreas	0/54	–	–	–	0/54
Liver	–	–	0/73	–	0/73
Stomach	–	0/54	0/185	–	0/239
Prostate	0/25	0/24	–	–	0/49
Colon	0/20	0/56	0/98	1/293	1/467
Kidney	0/15	–	–	–	0/15
Brain	0/4	–	–	0/58	0/62
Gall bladder	–	0/50	–	–	0/50
Bladder	–	0/28	–	–	0/28
Leukemia (adult)	–	–	0/88	–	0/88
Total	0/324	0/243	0/537	1/351	1/1455

Summary

The aims of tumor classification include estimation of clinical outcomes and tumor characteristics, choice of treatment based on such estimation, understanding of cancer pathogenesis, and so on. On this basis, it is very important to discriminate SCLC from NSCLC in current therapeutic strategies, which are based on consensus resulting from many studies describing differences in clinicopathologic features, response to therapy, and treatment outcomes. On the other hand, comparison of genome-wide expression profiles of lung cancers suggests that there is a great distinction between adenocarcinomas, namely TRU- and non-TRU-type adenocarcinomas. Evidence for this distinction is supported by specific involvement of *EGFR* mutation and different impacts of smoking in these two subsets. Further studies are warranted to clarify the distinctions between lung adenocarcinomas, and consideration of these differences may provide us with greater understanding of lung cancer pathogenesis and allow us to develop better therapeutic strategies.

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