Molecular profile of lung cancer in never smokers

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Tobacco smoking is the most common cause of lung cancer, but approximately 10–25% of patients with lung cancer are life-long never smokers. The cause of lung cancer in never smokers is unknown, although tobacco-smoke exposure may play a role in some of these patients. Lung cancer that develops in the absence of significant tobacco-smoke exposure appears to be a unique disease entity with novel genomic and epigenomic alterations and activation of molecular pathways that are not generally seen in tobacco-smoke-induced lung cancer. These molecular alterations are very likely responsible for the unique clinico-pathological features of lung cancer in never smokers (LCINS), and some of these molecular alterations – such as the activating *EGFR TK* mutations and *EML4–ALK* fusion – significantly influence therapeutic choices and treatment outcomes. In the last few years there has been a number of studies exploring the molecular characteristics of LCINS, and some of them have reported new and significant findings. Here we review the key findings from these studies and discuss their potential therapeutic implications.

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1. Introduction

Globally, over a million patients are diagnosed with lung cancer each year, making it the most common type of cancer in the world [1]. Even though tobacco smoking is considered to be the most common cause of lung cancer, it is estimated that 10–25% of all patients diagnosed with lung cancer are never smokers [2]. Never smokers with lung cancer are more likely to be women, have adenocarcinoma histology and are of East Asian ethnicity when compared to tobacco smokers with lung cancer [3–5] Apart from these now well-established epidemiological differences, recent research has uncovered several key molecular alterations that are more frequently detected in never smokers with lung cancer. Some of these molecular alterations – such as activating mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (*EGFR*) gene and the *EML4–ALK* fusion – have therapeutic relevance in the treatment of patients with advanced-stage lung cancer [6–10]. Comprehensive genomic analysis by whole genome sequencing has also identified significant differences between the tumour genome of lung cancer in never smokers (LCINS) and tobacco smokers with lung cancer [11] (Table 1). In this review we will discuss the genomic and epigenomic findings that characterise LCINS.

2. Inherited susceptibility to LCINS

Despite the fact that tobacco smoking is the primary cause of lung cancer, identification of familial clustering of patients with lung cancer is suggestive of an inherited risk factor. Several studies have reported that patients with LCINS are more likely to have a family member diagnosed with lung cancer than a tobacco smoker with the same disease [12–15]. A systematic review of 11 studies identified that a positive family history of lung cancer increases the risk of developing lung cancer by 1.5-fold in never smokers [16]. A linkage study of 52 families with two or more members diagnosed with lung cancer identified the 6q23–25 region to be a major

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Table 1 – Characteristic molecular variations in lung cancer in never smokers (LCINS).

	Markers	Lung cancer in tobacco smokers	LCINS
Genomic changes	Point mutations in protein coding regions	Primarily G → T transversions	Primarily G → A transitions
	KRAS mutation	Common, 30–43%	Rare, 0–7%
	EGFR mutation	Rare, 0–7%	Common, 45%
	TP53 mutation; G → T to G → A ratio	Ratio = 1.5	Ratio = 0.23
	STK11 mutations	14%	3%
	EML4-ALK fusion	2–3%	5–11%
	ROS fusion	<1%	1.5–6%
	RET fusion	<1%	2%
Epigenomic changes	Methylation index (MI)	High MI	Low MI
	p16 and *APC* methylation	Common	Rare
	Loss of protein expression in *hMSH2*	Less common, 10%	More common, 40%

susceptibility locus for lung cancer [17]. In addition, three large genome-wide association studies (GWASs) identified the 15q24–25.1 locus as the site harbouring genetic polymorphisms associated with lung cancer risk [18–20]. However, a pooled analysis of data from all three studies did not find the 15q24–25.1 locus to be associated with increased risk for LCINS [21].

Studies have also examined whether polymorphisms of genes involved in carcinogen metabolism, DNA repair and inflammation are associated with increased risk for developing LCINS. Pooled analysis of studies evaluating *CYP1A1* and *GSTM1* polymorphisms identified that *CYP1A1*-I462V polymorphism was associated with two- to three-fold increased risk for developing LCINS. Interestingly, the *CYP1A1*-I462V polymorphism was associated with increased risk for LCINS only in Caucasians, not in Asians [22]. However, these findings are limited by the small sample size of patients with LCINS in each individual study, and they were focused on a limited number of molecular alterations. Individual studies have shown specific polymorphisms involving DNA repair genes (*XRCC1* and *ERCC2*) and genes involved in interleukin production (IL1, IL6 and IL10) to be associated with increased risk for LCINS [23–25]. These studies are limited by their relatively small sample size and require independent validation to ascertain that these polymorphisms are associated with increased risk for LCINS.

3. Markers of tobacco exposure

Significant differences have been reported in the frequency and patterns of gene mutations between LCINS and lung cancer in tobacco smokers (reviewed in [26]). Some of the earliest studies identified that mutations in the tumour suppressor gene TP53 were less frequent in LCINS (8–47%) when compared with tobacco smokers with lung cancer (26–71%) [27–29]. Also a significant dose–response relationship between tobacco smoke and *TP53* mutations has been reported in patients with non-small-cell lung cancer (NSCLC) [27]. In a sample of 30 resected NSCLC tumor samples the odds of having *TP53* mutations in a patient smoking 20 cigarettes per day for 30 years were 5.3 when compared with a patient with LCINS. Tobacco-smoke exposure was also associated with a distinct mutational spectrum in the *TP53* gene, with increased frequency of G → T transversion mutations when compared to LCINS [30,31].

Mutations involving the *KRAS* oncogene are rare in patients with LCINS and are more frequently reported in tobacco smokers with lung cancer [32–36]. In a sample of 106 patients with adenocarcinoma, the incidence of *KRAS* mutations was significantly higher in the smokers cohort versus the never smokers (43% versus 0%, $P = 0.001$) [35]. Similarly *KRAS* mutations are more frequently identified in tobacco smokers and are predominantly G → T transversion mutations [31].

4. Fusions and mutations involving kinase genes

Analyses of tumor samples from patients with excellent response to treatment with EGFR TK inhibitors led to the discovery of activating mutations involving the *EGFR TK* gene [6,7]. At around the same time it was also discovered that patients with LCINS had a better response to EGFR TK inhibitors such as gefitinib [37]. Several retrospective studies subsequently established that patients with LCINS were more likely to harbour the *EGFR TK* mutation than tobacco smokers with lung cancer [8,38,39]. One of the largest studies ($n = 1082$) confirmed that activating *EGFR TK* mutations were more frequent in patients with LCINS than in tobacco smokers with lung cancer: 54% versus 16% [40]. The higher incidence of *EGFR TK* mutations in LCINS has been a consistent finding across different ethnic and geographical divisions. In addition, the frequency of EGFR TK mutations is inversely related to tobacco-smoke exposure. The proportion of *EGFR TK* mutations in patients with less than 20 pack year exposure was 55% versus 27% for 20–50 pack years and 22% for >50 pack years ($P < 0.001$) [38]. Pham and colleagues reported similar findings: decreasing incidence of *EGFR TK* mutations with increasing pack years [39]. The difference was significant when exposure was >15 pack years (9%) versus never smokers (51%); $P < 0.005$. In addition, *EGFR TK* mutations were not detected in tobacco smokers with more than 75 pack year exposure.

The *EGFR TK* inhibitor erlotinib was initially approved for the treatment of all patients with advanced NSCLC in the second- and third-line settings. The discovery of activating *EGFR TK* mutations led to several randomised trials comparing EGFR TK inhibitors with chemotherapy in the front-line

setting in patients with *EGFR TK* mutations [41–43]. Results from these trials have now established EGFR TK inhibitors as the standard front-line treatment for patients with advanced-stage NSCLC that is positive for *EGFR TK* mutation.

Mutations involving the *HER2* gene have been shown to be more frequent in never smokers with adenocarcinoma [9]. In a sample of 671 NSCLC tumours, the overall frequency of *HER2* mutations was low at 1.6% (11/671), but they were more frequently identified in never or light smokers (8 of 248, 3.2%; $P = 0.02$). The *HER2* mutations were not detected in tumours harbouring either the activating *EGFR-TK* or *KRAS* mutations.

The *STK11* gene encodes a serine–threonine kinase and plays an important role in cell proliferation and survival. Mutations involving the *STK11* gene have been reported in 8% of all patients with lung cancer. In addition, they are more frequently present in tobacco smokers with lung cancer than in patients with LCINS (14% versus 3%; $P = 0.007$) [44].

EML4–ALK is a novel fusion gene present in approximately 5% of patients with NSCLC and is associated with an excellent therapeutic response to treatment with an Alk kinase inhibitor [10,45,46]. The fusion gene was more frequently identified in never smokers and younger patients with lung cancer. In addition, it appears to be mutually exclusive to *EGFR TK* and KRAS mutations.

Two new transforming fusions involving the *RET* and *ROS1* kinase genes at the 3′ end have been identified in patients with lung cancer [47]. In one study, tumour samples from 936 patients with surgically resected NSCLC were tested for *RET* fusion genes by the reverse transcriptase polymerase chain reaction (PCR). The *RET* fusion was detected in 13 patients (1.4%), and these patients predominantly had adenocarcinoma histology (84.6%), were never smokers (82%) and many of them were younger: age $\leqslant$ 60 years at the time of diagnosis (73%) [48]. *RET* fusions have been shown to promote cell proliferation, and treatment with vandetanib, a multi kinase inhibitor with activity against *RET* kinase, was able to inhibit *RET*-induced cell proliferation [47]. Fusions involving the ROS1 gene in lung cancer were first reported in 2007 [49] and in a subsequent study, a fluorescent in situ hybridisation (FISH) based assay of 1000 NSCLC tumour samples identified ROS1 fusions in 18 (1.7%) samples [50]. Similar to patients with ALK or RET fusions, ROS fusions were found primarily in younger patients who were never smokers and had adenocarcinoma histology. Cell lines expressing ROS fusion were sensitive to treatment with the ALK inhibitor crizotinib. Overall, fusion genes involving the ALK, RET and ROS kinases are relatively rare molecular events in patients with NSCLC. These patients have similar clinico-pathological features, including that of being a never smoker. In addition, these fusions appear to be mutually exclusive to each other and to other known driver mutations in lung cancer, such as EGFR TK and KRAS mutations.

5. Epigenetic alterations

Methylation of tumour suppressor genes – including $p16^{INK4a}$, *DAPK*, *RASSF1A*, *RARβ*, *APC*, *CDH13*, *MGMT*, *hMLH1*, *hMSH2* and *GSTP1* – leading to epigenetic silencing has been reported in lung cancer (reviewed in [51,52]). Studies have reported that methylation of the tumour suppressor gene *p16* is less frequent in LCINS in comparison to lung cancer in tobacco smokers [53–57]. In a sample of 514 NSCLC tumours, which included 112 never smokers with adenocarcinoma, *p16* ($P = 0.007$) and *APC* ($P = 0.0007$) methylation rates were significantly lower in never smokers than tobacco smokers with adenocarcinoma [54]. There was no significant difference in the methylation rate of the other tumour suppressor genes *RASSF1A*, *RARβ*, *CDH13*, *MGMT* and *GSTP1* between the two groups. The methylation index (total number of genes methylated/total number of genes examined) was significantly higher in tobacco smokers with lung cancer when compared to LCINS. In a follow-up study of 383 NSCLC tumours, the authors confirmed that the *p16* methylation rate and the methylation index were significantly lower in LCINS ($P < 0.0001$) [55]. The methylation rate for *APC* was significantly lower ($P < 0.0001$) in never smokers when the analysis was restricted to adenocarcinoma. Subsequent studies have also reported a low *p16* methylation rate in never smokers with adenocarcinoma [56,58]. There was no significant difference in the methylation rates of *RASSF1A* and *DAPK* between tobacco smokers with lung cancer and LCINS [56].

The loss of protein expression in protein mismatch repair genes *hMLH1* and *hMSH2* was reported to be more frequent in LCINS than in lung cancer in tobacco smokers [59]. In a sample of 77 resected NSCLC tumours, the loss of protein expression for *hMLH1* (70% versus 46%) and *hMSH2* (40% versus 10%) was more frequent in LCINS. The authors also reported that promoter methylation was the predominant mechanism for the loss of protein expression in both genes.

6. Next-generation sequencing in LCINS

The advent of next-generation sequencing technologies now allows us unprecedented access to the tumour genome. Recently, next-generation sequencing of several tumour–normal pairs from patients with NSCLC was reported, and some of these patients were never smokers. Whole genome and transcriptome sequencing was performed in 17 patients with NSCLC, including five never smokers and 12 tobacco smokers [11]. The total number of mutations involving genes in protein coding regions was significantly higher in smokers than in never smokers; median 209 versus 18. In addition, the mutations in tobacco smokers were primarily G → T transversions, whereas in LCINS they were G → A transitions. For the first time this study identified that the G → A transition point mutations in never smokers is a genome-wide phenomenon and is not restricted to *KRAS* and *TP53* genes.

Genomic and epigenomic profiling of tumour–normal pairs from six Korean patients with LCINS with exome seq, RNA seq, micro RNA seq and methylated DNA immunoprecipitation-sequencing (MeDIP-seq) confirmed the low mutation rate in LCINS [60]. They reported a total of 47 somatic mutations from the six LCINS tumour samples. In addition, they identified several novel fusion genes, including *CCDC6–RET* fusion which has been previously reported and could be a potential therapeutic target. Pathway analysis identified that genes involved in cell cycle regulation – particularly in

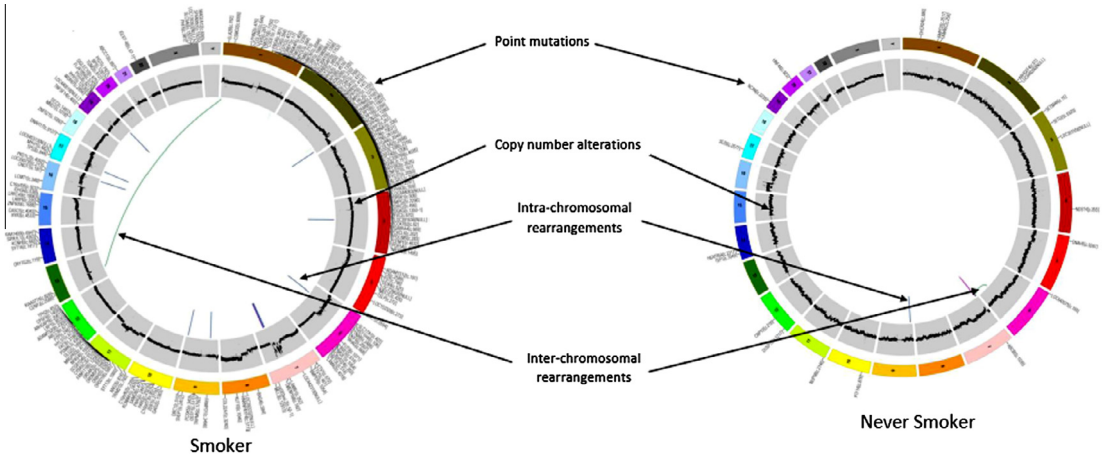

Fig. 1 – Circos plots of tumour genome from a never smoker with lung cancer and a tobacco smoker with lung cancer. Adapted from Govindan et al [11].

G2/M transition – are very likely to have played a significant role in the development of these tumours.

7. Conclusion

Cancer is a disease that is characterised by genomic and epigenomic alterations that result in malignant transformation of normal tissue. Such transforming genomic and epigenomic alterations are considered the drivers of the malignant disease and determine the clinical behaviour of the disease. In the case of lung cancer, tobacco-smoke exposure appears to be an important factor in determining the type of oncogenic drivers associated with the disease. This is well exemplified by findings from several studies showing that mutations involving *TP53* and *KRAS* genes are more frequent in tobacco smokers with lung cancer, whereas LCINS is characterised by *EGFR TK* mutations, *ALK*, *RET* and *ROS* fusions. The differences between LCINS and lung cancer in tobacco smokers are not restricted to a few genes. Recent next-generation sequencing studies have found that the genome of LCINS is significantly different from the tumour genome of a tobacco smoker with lung cancer (Fig. 1). Overall, the number of mutations is significantly lower in LCINS, and the point mutations are primarily G → A transitions.

The higher number of genomic alterations seen in smokers with lung cancer is very likely due to the mutagenic field effect of tobacco-smoke exposure. The vast majority of these genomic alterations in tobacco smokers with lung cancer are believed to be passengers that do not have any role in the malignant transformation or progression. In contrast, in LCINS the absence of tobacco-smoke exposure and the relatively smaller number of identified genomic alterations suggest that most if not all of them play a role in its malignant transformation. Hence the LCINS genome may provide us with a relatively enriched and easily identifiable set of oncogenic drivers for lung cancer. In addition, the relatively small number of genomic alterations in LCINS also presents better opportunities for the development of targeted therapies against LCINS. With the advances in sequencing technology and decreasing costs it is possible that, in the near future, advanced-stage LCINS may be primarily treated with molecularly targeted therapy, and it would be possible to achieve prolonged periods of disease control similar to the treatment of chronic myeloid leukaemia (CML) and gastrointestinal stromal tumour (GIST).

Conflict of interest statement

The author is not a government employee. For the last 2 years, he has been a consultant for Pfizer, Roche Genentech, Bristol-Myers Squibb, Merck, Boehringer-Ingelheim, Abbott Oncology and Covidien.

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