Epigenetics in Personalized Management of Lung Cancer

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Abstract In last several years, the focus on the origin and progression of human cancers has shifted from genetic to epigenetic regulation, with particular attention to methylation and acetylation events that have profound effect on the eventual expression of oncogenes and the suppression of tumor suppressors. A few drugs targeting these epigenetic changes have already been approved for treatment, albeit not for lung cancer. With the recent advances in the push towards personalized therapy, questions have been asked about the possible targeting of epigenetic events for personalized lung cancer therapy. Some progress has been made but a lot needs to be done. In this chapter, a succinct review of these topics is provided.

Keywords Epigenetics • Methylation • Acetylation • DNMT • HDAC inhibitors

1 Introduction

Lung cancer is a deadly disease that affects millions of lives worldwide. It is the leading cause of cancer-related deaths [1] and the rate of incidence is only predicted to go up in coming decades [2]. A number of targeted therapies have been evaluated to fight lung cancer, primarily targeting the various signaling pathways [2], and the results are far from satisfactory. In recent years, epigenetic events have gained attention based on the many reports that support a crucial role of these events in tumor progression.

Although the concept of epigenetics was first introduced by Waddington more than 70 years back in the year 1939 [3], it was not until about 30 years back when the first connection between epigenetics and cancer was noted in the year 1983 when lower DNA methylation and 5-methylcytosine levels were observed in human tumors, compared to normal tissues [4, 5]. A number of studies have emerged in the recent years that advocate the importance of epigenetics in the management of

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human cancers [6–9]. Epigenetics has been studied in the context of tumor progression [10], drug resistance, cancer stem cells (CSCs) [11–13], epithelial-mesenchymal transition (EMT) [14, 15] as well as the microRNAs (miRNAs) [16, 17]. There is also an indication for the use of nutraceuticals to bring about epigenetic changes, leading to improved cancer therapy [18, 19]. A number of studies have focused on epigenetics in lung cancer [20–23] which gives us enough information to ponder on how to use this information for an efficient clinical management of lung cancer patients.

Towards the advancement of personalized therapy, it is important to identify gene(s) or a gene signature that may pre-dispose individual patients to poor prognosis. For the early stage non-small cell lung cancer (NSCLC) patients, curative surgery is the primary strategy. A majority of these patients with resected cancer do well but about 30 % patients exhibit relapse and ultimately succumb to this deadly disease [24]. With this problem in mind, Harris and his coworkers at NCI worked on identifying a genetic signature that can help prognosis of NSCLC patients at particular risk of relapse and progression. In an initial report [25], four genes—BRCA1, HIF1A, DLC1 and XPO1 were identified as the gene signature associated with prognosis. The study involved cohorts from different geographical locations—US, Japan and Norway. As relevant to discussion on personalized medicine, this work suggests that stage I NSCLC patients undergoing curative surgery should further be tested for these four genes and, based on the test results, be monitored on a regular basis or appropriately recommended for adjuvant chemotherapy.

In a follow-up study on the four gene cluster above, a more robust meta-analysis was recently reported [24]. A total of 12 publicly available cohorts were selected for the analysis with 1069 stage I lung cancer patients. Geographical locations represented in this study were—US, Japan, Norway, Sweden, France and South Korea. The major finding of this analysis was that the patients identified as ‘high risk’, based on the expression of four gene signature, had worse overall survival. Since the patient cohorts had wide diversity in race and ethnicity, the results seem to be robust enough to be applied to almost any populations. One interesting observation from this study was that the gene signature was found to be prognostic only for adenocarcinoma. When the analysis was extended to squamous cell carcinoma, no prognostic value of this four gene cluster was observed. Regardless, these studies have helped identify a cluster of four genes that can help fine tune the post-operative management of a subset of early stage lung cancer patients which is a critical step towards personalized medicine.

2 Epigenetics: Promises and Challenges

A number of concepts have emerged in the last decade or so, such as targeting EMT or the CSCs or the miRNAs but nothing very concrete has come out with regards to targeted therapies. In contrast, epigenetics is blessed to have been evaluated/targeted in clinics through the use of several drugs that effectively target methylation

and histone acetylation [8]. Of all the different types of epigenetic changes, methylation and acetylation are the ones that have been studied in detail, with the aim of pharmacological intervention. Methylation is controlled by methyltransferases that increase methylation and the demethylases that decrease methylation. Acetylation is controlled by acetyl transferases that add acetyl groups (acetylation) and the deacetylases that remove acetyl groups (deacetylation). Acetylation generally leads to activation while deacetylation causes gene silencing [26]. Inhibitors of all these enzymes are being tested as anticancer therapies. This is ironical in a way, and exemplifies our evolving knowledge in the field. For example, inhibitors of histone deacetylases (HDACs), also called HDAC inhibitors, are one class of inhibitors that have gained a lot of attention with regards to their promise in anticancer therapy [27–29]. This is based on the concept that acetylation of genes is generally associated with reduced metastasis and reduced aggressiveness of cancers, and, therefore, deacteylation leads to increased metastasis and aggressiveness. It is thus conceived that inhibitors of deacetylases can work as anticancer agents by helping increase the acetylation of genes. While this concept has been tested well, through HDAC inhibitors such as trichostatin A and suberoylanilide hydroxamic acid (SAHA), it is interesting to mention that even the inhibitors of histone acetyltransferases (HATs), referred to as HAT inhibitors, have been proposed as promising anticancer agents [30, 31]. HDAC inhibitor trichostatin A has shown promise against small cell lung cancer [32] while HDAC inhibitors entinostat [26], CG200745 [33] have shown promise against NSCLC. In a study performed in prostate cancer model [34], we reported that HDAC inhibitors induced EMT and also enriched the CSC markers. This study indicated that HDAC inhibition might actually be counterproductive and may increase the cancer aggressiveness. While this may provide rationale for the mostly disappointing results with HDAC inhibitors in clinics, it also makes a case for more detailed studies on understanding the precise balance of acetylation and deacetylation in human genome.

An interesting aspect about epigenetic changes is that these alterations are reversible [35]. This is in contrast to genetic changes which are not. Being reversible, epigenetic changes present unique opportunity as therapeutic targets that can potentially be modulated in clinical settings. A case has also been made for combining epigenetic drugs to make a broader impact but this mostly comes at a cost—increased toxicity and off-target effects. Studies to combat this are in progress and there are early indications that novel drugs with pleiotropic effects against DNMTs and HDACs might be the way to go [36].

3 Methylation

Methylation is one of the better studied epigenetic event [37]. It was the first identified epigenetic mark [38]. In the methylation of DNA, a methyl group is transferred to the cytosine nucleotide within the DNA. The most vulnerable sites for such methylation are the CpG islands i.e. cytosines that are immediately followed by

guanines. Increased methylation (hypermethylation) leads to silencing of genes and, conversely, reduced methylation (hypomethylation) results in increased expression of genes. In a bioinformatics study that aimed to predict lung cancer, methylation of CpG islands was found to be the most important feature that influenced the predictive power [39] and methylation of CpG island has been proposed as a biomarker for early detection of lung cancers [40]. As mentioned above, methyltransferases add methyl groups while demethylases remove methyl groups. Thus, there is a dynamic process in place where methylation and demethylation events are synchronized to bring about the silencing and expression of genes, as needed. The main reason methylation is connected to gene silencing is because methylation makes genes inaccessible to cellular transcriptional machinery. Recently, DNA methylation patterns have been proposed as predictors of breast cancer [41] which makes it a valid assumption that similar predictors might be possible for lung cancer as well.

Three methyltransferases particularly involved in a bulk of methyl transfers are DNMT1 (DNA MethylTransferase-1), DNMT3A and DNMT3B. These DNMTs are attractive targets for drugs that affect methylation. A number of DNMT inhibitors are known, and some of them have been approved by FDA for treatment of specific cancers. Examples are 5-azacytidine and decitabine. EZH2 is another methyltransferase that adds methyl groups to its target histone H3 leading to suppression of tumor suppressors, which, in turn, leads to tumor progression and metastasis. Consequently, targeting of EZH2 in cancer therapy has been advocated [42–44]. EZH2 expression has been linked to acquisition of cancer stem cell properties and aggressive phenotype [45], thus validating its targeting for the treatment of human cancers. In NSCLC model, inhibition of epigenetic activity of EZH2 has been demonstrated to sensitize BRG1 and EGFR mutant lung cancers to inhibitors of topoisomerase II [46]. KMT1E/SETDB1 is yet another methyltransferase that methylates histone H3 and silences several genes. However, in contrast to EZH2, it is a tumor suppressor [47] that inhibits invasive and metastatic potential of lung cancer cells when over-expressed, and is significantly down-regulated in highly metastatic lung cancer cells. So, right here, we notice a conflict where one methyltransferase is oncogenic while the other one is a tumor-suppressor. EZH2 is comparatively more widely studied methyltransferase and we need to wait on more reports on KMT1E/SETDB1 to be able to make more sense of targeting these particular methyltransferases for the personalized treatment of lung cancers.

In addition to methylation of DNA, epigenetic changes also include methylation of histones wherein methyl group is transferred to individual amino acids in the histone proteins, for example, methylation of lysine 9 or lysine 27 in the histone H3 leading to H3K9me2/3 and H3K27me3 respectively. These methylations also lead to gene repression. There is also evidence of methylation leading to gene activation, such as the one seen in H3K4me3 [48]. While inhibitors of DNMTs were the first off the block, inhibitors of histone methyltransferases (HMTs) are also now being evaluated against many cancers [49].

Personalized treatment of lung cancer patients involving targeting of methylation, thus, comes across as a valid approach. In a cell line based study [50] that involved generation of highly aggressive lung cancer cells in vivo, a comparison of

methylation status of parental vs. the derivative highly metastatic cells revealed significant changes in the DNA methylation. Inhibition of DNMT, by azacytidine, reversed the metastatic phenotype, thus confirming the important role of DNA methylation in the metastatic potential in a lung cancer model.

4 Hypomethylation

As a proof that reduced methylation, or 'hypomethylation', causes over-expression, it has been reported that oncogenic KCNN4's promoter is hypomethylated and, therefore, KCNN4 is over-expressed in aggressive NSCLC cells [51] which also makes it a strong predictor for poor prognosis. Hypomethylation was also found prevalent in tumors, compared to adjacent non-malignant lung tissues, in a study that looked at lung cancers in non-smokers [52]. Tumors were found to be typically hypomethylated which would suggest over-expression of many oncogenes. Such differential methylation was particularly concentrated at CpG sites, although non-CpG sites were also found to be differentially methylated. ELMO3 (engulfment and cell motility 3) is another oncogene that was found to be hypomethylated, consistent with its over-expression in primary tumors from patients with distant metastases [53]. As suggested by these recent reports, it is evident that a role of reduced methylation, leading to induced expression of oncogenes, is increasingly being realized. From the perspective of personalized therapy, hypomethylation will need to be countered with increased rate of methylation, or the use of inhibitors that can target demethylases. This is rather a novel area of research as most of the focus has been on the inhibitors of methyltransferases. With the expansion of our knowledge on this subject, it would be interesting to evaluate findings with inhibitors of demethylases.

5 Hypermethylation

Hypermethylation is a more widely studied epigenetic event. As discussed by Barrow and Michels [54], there is evidence for hypermethylation of specific genes that can potentially be used for early diagnosis of lung cancer or for the identification of individuals at particular high risk. For example, methylation of three genes (*CDKN2A/p16*, *DAPK*, and *RASSF1A*) in sputum, conferred a significant increase in risk (OR > 1.5), particularly in smokers [55]. To test if such hypermethylation can be an early predictive event, analyses of sputum collected 18 or 19–72 months prior to diagnosis were performed. In samples collected within 18 months of diagnosis, a 6.5-fold increase in the risk of lung cancer was observed in individuals with hypermethylation of at least three genes from a six-gene panel (*CDKN2A/p16*, *DAPK*, *RASSF1A*, *GATA5*, *MGMT* and *PAX5 β*). However, 36 % controls also had similar hypermethylation. In samples collected 19–72 months before diagnosis, hypermethylation of just one gene (*CDKN2A/p16*) conferred an 80 % increased risk of

developing cancer. Further, another study reported hypermethylation of *CDKN2A/p16* and *RASSF1A* in only 1 of 18 sputum samples [56], thus putting a question mark on the utility of *CDKN2A/p16* and *RASSF1A* hypermethylation as biomarkers. A meta-analysis of 18 studies did identify hypermethylation of *CDKN2A/p16* as predictive of reduced disease-free survival [57]. Combined, it is evident that more detailed studies are needed to establish a role of hypermethylation of select genes as markers for lung cancer.

Multiple reports have found a connection between hypermethylation and reduced expression/silencing of genes. For example, β-catenin [58], CDH13 [59] and MARVELD1 [60] were reported to be epigenetically silenced through hypermethylation in lung cancers. Liu *et al.* reported hypermethylation of tumor suppressor TMEM196 [61] which was consistent with its reduced expression leading to aggressive lung cancer. Hypermethylation was found in close to two-thirds primary lung tumors and correlated with shortened survival, poor differentiation and pathological stage. Such hypermethylation of CpG islands has been linked to prognosis in stage I lung adenocarcinoma in an independent study [62]. In another example of hypermethylation-mediated suppression, NPTX1 was found to be hypermethylated at its promoter in lung cancer cells [63]. Hypermethylation was also observed in neoplastic human lung specimens, which correlated negatively with the mRNA levels of NPTX1. The overall survival time was significantly reduced in patients that harbored hypermethylated NPTX1. Li *et al.* [64] found evidence of hypermethylation of SOX1 in two-thirds of human lung cancer specimens which contrasted with increased methylation in just a quarter of adjacent normal lung tissues. As expected, methylation inversely correlated with SOX1 expression in NSCLC cells. Interestingly, an association between smoking and promoter hypermethylation has also been observed [65].

6 Epigenetics in Drug Resistance of Lung Cancers

Resistance to standard therapies is a major clinical problem. In recent years, some evidence has emerged that establishes a connection between epigenetic events and resistance to therapies. As an example, reduced methylation and resulting overexpression of MEOX2 correlated with chemoresistance in lung cancer patients [66]. HDAC inhibitors SAHA and ST3595 were found to significantly reduce the aggressive phenotype of cisplatin-resistant NSCLC cells A549 and H460 [67]. This was attributed to the ability of HDAC inhibitors to up-regulate tumor suppressor KiSS1. In a study that used paired cell lines—parental and doxorubicin resistant lung cancer cells A549, a number of epigenetic markers were found to be differentially expressed [68]. These included reduced levels of HDACs, DNMT and acetylated histones in the resistant cells, relative to parental cells. Trichostatin A and 5-aza-2′-deoxycytidine, the epigenetic modifiers re-sensitized resistant cells to doxorubicin, thus validating an important role of epigenetic modifications in the acquisition of

drug resistance in lung cancer cells. Epigenetic changes can modulate sensitivity to docetaxel as well because DNA methylation and resulting suppression of tumor-suppressor DKK3 (Dickkopf-related protein 3) has been linked to docetaxel resistance in NSCLC cells [69].

Although these reports are indicative of a role of epigenetic changes in drug resistance, and also point to the possibility of developing a personalized plan for the effective reversal of epigenetic changes for the successful 're-sensitization' to available therapies, it is important to note that the information on the subject is still evolving and more robust studies need to be planned. This notion is highlighted by a recent study that observed no-to-minimal utility of using epigenetic drugs as sensitization agents in different models representing NSCLCs [70].

7 Epigenetic Epidemiology

Another interesting concept that can be exploited in relation to personalized therapy of lung cancer is the study of epigenetic epidemiology [54]. This is a study of correlations between epigenetic variations and the risk of cancer within populations. The central concept is that population-wide analyses can help identify epigenetic changes that can predict either the onset of particular cancers or even resistance to therapies. For example, in lung cancer, tobacco smoke and air pollution are known risk factors that can influence DNA methylation. There is evidence of hypomethylation of *F2RL3*, *AHRR* and two intergenic regions smokers' blood, compared to non-smokers [71, 72]. *AHRR* stood out as a gene that significantly hypomethylated. It was interesting to note that hypomethylation could be detected in former smokers, albeit at a lesser degree, which is suggestive of a possible use of this gene as long-term marker of exposure [54]. Air pollution can also influence hypomethylation, as evident by hypomethylation of LINE-1 elements in leukocytes when exposed to black carbon and $PM_{2.5}$ [73]. Since tobacco smoke and air pollution represent high risk factors for lung cancers, understanding how they affect the epigenome will be important for early diagnosis of the disease. Such studies with broader populations can potentially yield some interesting results but are also prone to inconclusive findings, given the heterogeneity among individuals. They might also not be relevant to personalized therapy just because of the wider appeal, as opposed to focus on an individual patient.

8 Personalized Epigenetic Therapy: A Reality Check

As discussed in the preceding sections, we are slowly but surely realizing the big impact that epigenetic events seem to have on the onset, progression and outcome of lung cancers. However, it is still too premature to imagine a patient on a therapeutic

path, based on that patient's individual epigenome. There are many challenges that need to be overcome. The first and foremost is the mapping of patient's epigenome and the need to decipher all the unique epigenetic signatures that the patient presents. As might be evident from the discussion here, methylation and acetylation represent two better understood epigenetic events. However, there are several more epigenetic events that have although been recognized, such as ubiquitination, phosphorylation, sumoylation etc., but not necessarily studied in relatively detail. Many methodologies are emerging that have enabled high throughput analyses, again mostly focused on methylation and acetylation, but they do have associated economic barriers. Even when we go beyond this challenge, the next and even more formidable hurdle is the lack of targeted therapies to reverse the epigenetic changes for effective enforcement of personalized therapy. This is something that will only get better with more detailed studies. Clearly this area of cancer research is still in its infancy. Data from mostly in vitro studies is emerging with some encouraging validations in lung tumor specimens. The missing connection between these preclinical studies and the future clinical trials is the lack of appropriate in vivo models, although there seems to be some progress on that front too [74]. In an effort to make targeting of epigenetic events a part of personalized therapy, the next few years will be crucial. We will hopefully see more mechanism-based studies that will help establish clear marker sets that can eventually be tested as predictors or biomarkers.

9 Conclusions and Perspective

Enormous advances in last few years, like for example the next-generation DNA sequencing have aided in the evaluation of epigenetic events that accompany diseases, including cancer. We have seen an exponential increase in the research publications on the topic of epigenetics in cancer in recent years. By all indications, this area of research is not going to slow down. Several epigenetic therapies have been approved by FDA [75] and many more are in the pipeline. These advances, particularly in clinical trials, only verify the big potential of epigenetics in current cancer research because we clearly do not have a final word on how the epigenetic events are finely tuned, and, more importantly, how can they be manipulated by therapeutic interventions for the benefit of patients in clinics. While 'modifying' epigenome, it is important to recognize that epigenetic changes are rather global and are inherently associated with off-target effects, and the resulting toxicity. A tight regulation of epigenetic events can be achieved by targeting epigenetic enzymes to specific loci through the use of DNA-binding proteins such as zinc finger proteins (ZFPs), transcription activator-like effectors (TALEs) and clustered regularly interspaced short palindromic repeats (CRISPRs) [49]. This is an interesting concept and results from pre-clinical and clinical studies will be eagerly awaited.

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