ORIGINAL ARTICLE

A prospective study of association of cancer stem cell marker aldehyde dehydrogenase 1 with clinicopathological profile in lung carcinoma patients

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ABSTRACT

Context: In India, lung carcinoma is the fifth-most common tumor and second-most common tumor in the males as per the Indian Council of Medical Research registry of 2002. It has been seen that ALDH1 expression in non-small cell lung cancer (NSCLC) and the presence of marker was linked to a more tumorigenic potential in the *in vivo* assessment and shorter disease-free survival in NSCLC patients with platinum treatment. **Aims:** Hence, our objective was to detect association of cancer stem cell (CSC) marker aldehyde dehydrogenase 1 (ALDH1) with clinicopathological profile in lung carcinoma patients. **Settings and Design:** This is a Pilot study. **Subjects and Methods:** It was a Pilot study where biopsies from 55 fresh previously untreated lung cancer patients visiting the Pulmonary Medicine Department of Era's Lucknow Medical College and Hospital Lucknow and King George's Medical University were taken for 18 months November 2014–April 2016, after taking proper informed consent from them. Paraffin blocks were taken and stained by hematoxylin and eosin (Sigma) to make the histopathological diagnosis and immunohistochemistry was done for detection of CSC marker ALDH1 (Daco). **Statistical Analysis Used:** The statistical analysis was done using Statistical Package for Social Sciences Version 15.0 Statistical Analysis Software. The values were represented in number (%) and mean ± standard deviation. **Results:** Expression of stem cell marker ALDH1 with the staging of the tumor was observed in 62.5% of Stage I, 80% of Stage II, 94.1% of Stage III, and 100% of Stage IV cases. Statistically, there was a significant association between ALDH1expression and stage of disease ($P < 0.001$). Diagnostic efficacy of ALDH1 expression in the detection of any positive clinical stage, it was found to be 88.6% sensitive and 90.9% specific. **Conclusions:** Strong ALDH1 expression correlates with higher stage of lung carcinoma making it a prognostic marker needing in-depth study.

KEY WORDS: Lung cancer, staging, stem cells

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INTRODUCTION

In India, lung carcinoma is the fifth-most common tumor and second-most common tumor in the males as per the Indian Council of Medical Research registry of 2002. It accounts for 6.9% of new cancer cases detected each year.[1] The absolute and relative frequency of lung cancer has risen dramatically over the decade. An example of the increasing frequency of lung cancers is that in the 19th century the age-adjusted death rate from lung cancer was similar to that of pancreatic cancer; however, a worldwide increase has been seen since then in deaths caused by Lung carcinoma leading to increased age-adjusted death rates. In 1985, Lung carcinoma became the leading cause of cancer deaths in women and now causes approximately twice as many deaths as breast cancer. Of late as per WHO Lung cancer deaths are declining in men, and the death rate in women has plateaued secondary to decreases in smoking.[1] Still, it is seen that more women die annually of lung cancer than breast cancer which could be attributed to the increasing trend of smoking and passive smoke inhalation in women.[1] However, 9.3% of cancer-related deaths in both the sexes is still attributed to lung cancer.[2]

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Despite a number of factors like the increasing numbers of new cases (171,600) per year, the large numbers of current and ex-smokers at risk in the United States (50 million each), position of lung cancer as a predominant cause of death among Americans (6%), and a constant 72% mortality rate among lung cancer patients, there is almost no strategy or research funding for this epidemic even in the developed world.[3] Its status as a killer is worse in developing countries like India.

The major cause of development of such malignancy is the alkaloids and carcinogens present in the cigarette or bidi smoke being inhaled by the smokers as well as their family members passively, leading to predominantly squamous or the small cell variety of tumors. Other factors being implicated in pathogenesis of lung carcinoma are compounds such as arsenic, asbestos beryllium, coal, coal tar, and radon due to indoor exposure gamma radiations. Smoking leads to the development of squamous cell carcinoma which is more central or hilar in position and prone to metastasize. The response to chemo-radiation in this type of tumor is not very good and is with a poor outcome. Lung cancer is a cause of concern because, on one hand, where the management of breast, cervical, and prostate cancer has seen considerable progress which has led to improved survival rates, mortality from lung cancer has remained largely unchanged with the best reported 5-year survival rates for lung cancer being a mere 10%–15%.[4]

Patients can live with undetected lung cancer for years before it becomes apparent. Early lung cancer is largely asymptomatic, and because of internalization of tumors, the patients are not alerted by obvious physical changes. Squamous cell carcinoma takes around 8 years to reach a size of 30 mm when it can be commonly diagnosed so, by the time symptoms arise, the risk of metastasis is considerable.[5,6] Once symptoms appear they are often ignored by patients, delaying the diagnosis and treatment even further. The reasons for the patient delay in diagnosis are poorly understood.

The high mortality of lung cancer is very largely because approximately 80% of patients with lung cancer have Stage III or IV disease at presentation and are beyond therapeutic resection and care.[7] Ample evidence of a prolonged preclinical phase in lung cancer has been seen. Detection of the tumor at an earlier stage improves the 5-year survival to around 60%.[6] It is proven that the earlier the lung cancer is discovered, the better are the patient's chances of survival. Patients with radiograph-documented stage I lung cancer have a 5-year survival rate of 40%–80%, whether discovered by screening or accident. However, mortality in lung cancer worsens rapidly with advancing stage at the time of diagnosis.[8]

Histopathological diagnosis is necessary in lung cancers to rule out the possibility that the disease is not a nonmalignant process, to determine whether a lung cancer is a non-small cell lung cancer (NSCLC) or an SCLC and to differentiate a lung metastasis from a primary lung tumor. The most common tumor in smokers is the squamous cell carcinoma and small cell carcinoma. Another variety is the glandular tumor, adenocarcinoma which is more commonly occurring in women, subpleural or more peripheral in position. Atypical glandular proliferation is another premalignant condition showing no association with smoking. It has been seen that there are clones of endobronchial cell populations which accumulate genetic mutations leading to more malignant and finally an invasive malignant state.

The need of the hour in the field of lung cancer research is discoveries which will lead to the development of new detection technologies, identification of key risk factors, and/or the validation of interventions for precancer or early cancer. A multimodal approach may be required to optimize early detection and management of lung cancer from screening programs and early attempts at this approach look promising.[9] A large number of diagnostic modalities are available in diagnosing the lung carcinomas. For example, cytological examination of sputum, specimens collected at bronchoscopic examination such as bronchial washing, lavages, tracheobronchial, and transthoracic fine-needle aspiration and histology examination of bronchial biopsy and open lung biopsy. Despite the wide range of tests available, the delay in patient diagnosis could be due to many factors, such as socioeconomic status, cultural differences, and health-care differences, and shortcomings. Similarly, the reasons for late detection of tumor and delayed therapy are that clinically the primary symptoms are too nonspecific to raise alarm.[10] Hence, in this context, a study found that the time from onset of symptoms to treatment was shorter in patients with Stage IV lung cancer (median 3.4 months) than in those with Stage I/II disease (median 5.5 months).[11]

According to popular newer concepts, cancer stem cells (CSCs) are a rare population of undifferentiated cells driving tumor initiation, maintenance and spreading.[11,12] Most therapies are directed at the bulk of rapidly dividing tumor cells but not the slow dividing CSCs. Expression of the stemness factors results in genetic plasticity that allows these cells to remain in a dormant, drug-tolerant state. Eradicating CSCs, in addition, or instead of the fast-growing tumor mass seems to constitute a promising approach to achieve a long-lasting response and thereby to improve cancer therapy. A large body of evidence is in favor of the CSC concept; still, several aspects of its foundations were questioned. For example, the proof and enumeration of CSCs in xenograft transplantation experiments is subject to the degree of immune system incompetence of the host and appropriate microenvironmental conditions.[13,14]

The measurement of aldehyde dehydrogenase 1 (ALDH1) activity and expression represents a universal marker for the identification and isolation of CSCs from multiple sources.[10] In human lung cancer, cell lines ALDH1 activity has been seen to be associated with self-renewal and differentiation, resistance to chemotherapy, expression of CD133 and enhanced tumorigenicity, as well as ability to recapitulate the original tumor heterogeneity *in vivo*.[11] Aldehyde dehydrogenase and its isoforms are responsible for

acetaldehyde oxidation and control the differentiation of normal stem cells.[11] ALDH1 has been suggested as the specific marker for human lung adenocarcinoma.[11] Unlike CD133, increased CSC ALDH1 has been found to be helpful in tumor staging and providing prognostic information. Furthermore, lung cancer cells that expressed ALDH1 were shown to be highly tumorigenic and clonogenic in addition to being capable of self-renewal in comparison to lung cancer cells that do not express ALDH1. These insights suggest that ALDH1 functions in selecting for a subpopulation of self-renewing NSCLC stem-like cells have a greater possibility of being tumorigenic.[15]

It would be interesting to study the presence of CSC ALDH1 in lung cancer a tumor mainly graded by ALK and EGFR expression till now. Furthermore, it would be interesting to see if any correlation exists between the expression of these markers in the tumor and clinical staging of the disease at the time of diagnosis. This is an attempt to study the presence of CSC ALDH1 in lung cancer and correlate it with clinical outcome.

SUBJECTS AND METHODS

Population study

A total of 55 patients visiting Era's Lucknow Medical College and hospital and King George Medical University, suspected to be suffering from lung cancer were taken in this pilot study for 18 months November 2014–April 2016. Patient consent and ethical clearance were taken before the study was conducted. Patients with any comorbidity such as TB, fungal infections, or endocrine diseases were excluded from the study. Lung tissue fixed in 10% formalin processed in paraffin blocks, used for staining, sigma for hematoxylin and eosin staining and Protein Tech for Immunohistochemistry. The slides were examined in Olympus Penta-head microscope. Cases which were newly diagnosed and had no other associated malignancy or histories of chemo or radiotherapy were taken.

Immunohistochemistry and scoring

Immunohistochemistry (IHC) was done using the protein tech kit supplied. The tissue was first deparaffinized and then rehydrated, and then primary antibody was applied followed by application of enzyme-conjugated secondary antibodies. After adding, the substrate-specific staining was visualized. If weak or no staining was observed, an antigen' unmasking' enzyme digestion was required.

No specific scoring system for the two markers under study, ALDH1 has been designed which can be considered diagnostic as is the case with ER PR in breast carcinomas. However, in one study, done on oral lesions using the same markers, the authors designated the following method of scoring: the membrane and/or cytoplasmic immunoreactivity in epithelium of oral lichen planus (OL) was considered to indicate ALDH1-positive expression. ALDH1 was classified into two categories to make analysis of their prognostic values for cancer development. According to Cioue *et al*.'s scoring of % cells staining they classified staining in <5% and >5% of epithelial cells of OL as ALDH1 negative and positive, respectively.[7] This is the method adopted by us for designating our sample as positive or negative.

Statistical analysis

The statistical analysis was done using Statistical Package for Social Sciences, Lucknow, India Version 15.0 Statistical Analysis Software. The Mean, standard deviation Student's *t*-test ANOVA Kruskal–Wallis tools were applied for statistical analysis.[12]

RESULTS

Age of patients ranged from 35 to 72 years. Majority of patients were aged above 50 years (54.5%). Mean age of patients was 53.51 ± 10.76 years. Majority of cases were males (42 cases) (76.4%). There were 13 (23.6%) females. Male to female ratio of patients was 3.23:1 [Table 1]. SI/FQ Smoking index or frequency ranged from 10 to 60 with a mean value of 25.47 ± 13.09 [Table 2]. A total of 13 (23.6%) cases had no ALDH1 expression. A total of 4 (7.3%) had Score 1, 5 (9.1%) had score 2, and majority (n = 33; 60%) had Score 3. For Stage 0, ALDH1 expression was observed in only 3 (27.3%) cases. The expression of ALDH1 was observed in 62.5% of Stage I, 80% of Stage II, 94.1% of Stage III, and 100% of Stage IV cases. Statistically, there was a significant association between ALDH1 expression and stage of disease ($P < 0.001$) [Tables 3 and 4]. On evaluating the diagnostic efficacy of ALDH1 expression in the detection of any positive clinical stage, it was found to be 86.4% sensitive and 72.7% specific. The positive and negative predictive values of expression were 92.7% and 57.1%, respectively. The overall accuracy of the method was 83.6%. For stage 0, 72.7% patients had score 0 and 1 (9.1%) had score 2 and 2 (18.2%) had score 3. Mean score for stage 0 was 0.64 ± 1.21. For Stage I, only 37.5% had score 0, 2 (25%) each had score 1 and 2 and 1 (12.5%) had score 3. Mean score was 1.13 ± 1.13. For Stage II, 20% had score 0, 20% had score 2, and 60% had score 3. Mean score was 2.20 ± 1.23. For Stage III, none had had score 0 or 1, 5.9% had score 2, and remaining 94.1% had score 3. Mean score was 2.94 ± 0.24. For Stage IV, none had score 0, 11.1% had score 1, and remaining 88.9% had score 2. Mean score was 2.78 ± 0.67. Statistically, a significant association between ALDH1 expression score and clinical stage was observed ($P < 0.001$) [Figures 1a-d].

DISCUSSION

The predominant reason for high mortality rate in lung cancer patients is early tumor spread of lung cancer cells to distant, metastatic sites, and primary or acquired resistance of those cells to systemic therapy. Consecutively, more than two-thirds of the patients are diagnosed with locally advanced or metastatic disease and nearly half of the patients who are diagnosed with early-stage disease relapse within 5 years after surgical removal of the tumor mass and succumb from widely spread therapy-resistant disease.[16]

There is a growing body of evidence that CSCs represent rare population of exclusively tumorigenic cells responsible for tumor

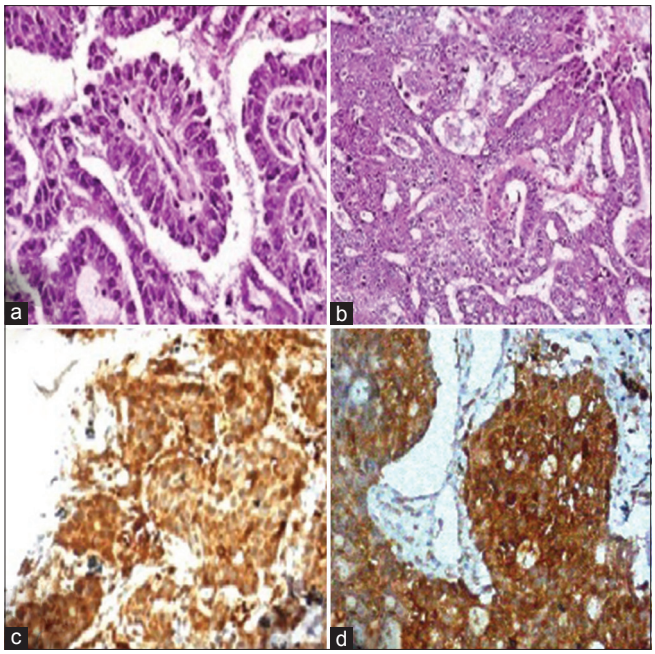

Figure 1: (a) Adenocarcinoma lung (H and E, ×40). (b) Adenocarcinoma lung (H and E, ×10). (c) Aldehyde dehydrogenase 1-strong cytoplasmic positivity (immunohistochemistry, ×40). (d) Aldehyde dehydrogenase 1-strong positivity (immunohistochemistry, ×40)

initiation, progression, metastasis, and recurrence.[17,18] Therefore, a better understanding of the biology of CSCs is providing opportunities for improved cancer detection and therapy in future. Various markers have been proposed to define stem cell populations in distinct solid tumors types. Expression of high ALDH1 enzymatic activity is a well-accepted markers for lung CSCs. Both markers independently allow for selection of cells that have the ability to self-renew, to initiate tumors when transplanted into SCID mice, and to differentiate into nontumorigenic cells, which form the bulk tumor mass.[19-21]

We studied the presence of CSC ALDH1 in lung cancer biopsies of newly diagnosed cases and correlated them with stage and other clinic-demographical features. Statistical analysis was done using Chi-square test, Student's *t*-test, with a comparison between parameters done using ANOVA and Kruskal–Wallis H-test. On studying the demographic data of the cases, we found that the age of patients under study ranged from 35 to 72 years. The majority of patients were aged above 50 years (54.5%) with the mean age of patients being 53.51 ± 10.76 years. In the studies reviewed the mean cutoff age of patients taken was 40 years,[22-24] while another study put the cutoff age as 56 years.[25] Another study showed the cutoff age as 67 years with age ranging between 37 and 82 years, with 40% of the total cases being above 67 years of age. They also studied the relation of CSC with age and has reported an increased expression of CD133 with age.[26] However, no such trend was noted in our study. Studying the sex distribution and exposure to risk factors, of patients under study we saw that majority of cases were males (42) (76.4%) while there were 13 (23.6%) females with the male to female ratio of patients being 3.23:1. Our finding was

Table 1: Demographic profile of the cases (*n*=55)

Characteristic	*Statistic (%)*
Age (years)	
31-40	10 (18.2)
41-50	15 (27.3)
51-60	17 (30.9)
61-70	12 (21.8)
>70	1 (1.8)
Mean age±SD (range) in years	53.51±10.76 (35-72)
Gender	
Male	42 (76.4)
Female	13 (23.6)

SD: Standard deviation

Table 2: Exposure to different risk factors and pattern of exposure

Characteristic (55 cases)	*Statistic (%)*
Smoke	
Smoking	42 (76.4)
Biomass	11 (20.0)
Biomass + smoking	2 (3.6)
Chewing tobacco/gutka	8 (14.5)
Mean duration of smoking±SD (range) (*n*=43)	18.58±8.28 (10-45)
Mean SI/FQ±SD (range) (*n*=43)	25.47±13.09 (10-60)

SD: Standard deviation; SI/FQ: Smoking index/Frequency

Table 3: Association between clinical stage and aldehyde dehydrogenase 1 expression

Clinical stage	*Total number of cases*	*Expression (%)*	*No expression (%)*
Stage 0	11	3 (27.3)	8 (72.7)
Stage I	8	5 (62.5)	3 (37.5)
Stage II	10	8 (80)	2 (20)
Stage III	17	16 (94.1)	1 (5.9)
Stage IV	9	9 (100)	0

Table 4: Prognostic efficacy of aldehyde dehydrogenase 1 expression for Carcinoma lung

CD133 expression	*Clinical Stage I or above*	*Clinical Stage 0*	*Total*
Present	38	3	41
Absent	6	8	14
Total	44	11	

Sensitivity: 86.4%; Specificity: 72.7%; PPV: 92.7%; NPV: 57.1%; Accuracy: 83.6%. PPV: Positive predictive value; NPV: Negative predictive value

supported by another study done by Mohan *et al*. where they saw that the ratio of men to women was 7.4:1.[24] In another study, the male to female ratio was found to be increased (3.58:1) with 78% of the total population being males.[25]

It has been seen in a study that the prevalence of CSC marker ALDH1 was more in NSCLC than SCLC and that high levels of ALDH1 correlated with tumour stage, grade, and poor prognosis.[25,26] In a study done on oral cancers using ALDH1 in serial tissue sample of OL out of the 141 cases, 54 (38.3%) showed positivity for ALDH1 while and 32 (22.7%) showed positive expression for CD 133[27] a finding that matches our study where on studying presence or expression of stem cell markers ALDH1 in all the 55 cases a total of 76.4% showed positivity for ALDH1.

Wang *et al.*[28] reported that positive ALDH1A1 staining was detected in 41.28% (45/109) of the cases and ALDH1A1 mRNA expression was markedly elevated in most tumor tissues compared with adjacent normal tissues. In a study done by Miyata *et al.*,[29] they found the mean of 92 cases expressing ALDH1 on IHC as 52.1 which was significant expression On analyzing the scoring for ALDH1 we found that 13 (23.6%) cases had no ALDH1 expression. A total of 4 (7.3%) had Score 1, 5 (9.1%) had score 2, and majority (n = 33; 60%) had Score 3. Miyata's study in 2016 shows a higher of scoring in poorly differentiated tumors for both the markers.[29]

On studying the association between clinical stage and ALDH1 expression, ALDH1 expression was observed in only 3 (27.3%) cases of stage 0. The expression of ALDH1 was observed in 62.5% of Stage I, 80% of Stage II, 94.1% of Stage III, and 100% of Stage IV cases. Statistically, there was a significant association between ALDH1 expression and stage of disease ($P < 0.001$). On studying the Association of ALDH1 Expression Score with clinical staging statistically, a significant association between ALDH1 expression score and clinical stage was observed ($P < 0.001$). On studying the prognostic efficacy of ALDH1 expression for Carcinoma lung, it was found to be 86.4% sensitive and 72.7% specific. The positive and negative predictive values of expression were 92.7% and 57.1%, respectively. The overall accuracy of the method was 83.6%. Miyata *et al.* have also reported a higher scoring of the marker in higher stage of tumour.[29] However, as far as the expression of ALDH1 is concerned, it has been seen that isolated ALDH1 positive cells exhibit tumorigenic potential in both *in vivo* and *in vitro* studies and that immunohistochemical analysis of ALDH1 in lung tumour samples has shown positive correlation with tumour stage, grade, and poor tumor prognosis hence proving our findings of higher expression (score 3) in higher stages to be true.[26,30,31] However, no significant correlation was seen with marker expression and histological subtype, nuclear grade (especially in adenocarcinomas) and tumor inflammation in other studies done using the markers ALDH1 and CD133.[26,32]

ALDH1 has been seen to be associated with more aggressive tumor behavior and poor survival in NSCLC.[33] Miyata *et al.*[29] in their study, 2016 saw that high ALDH1 score was associated with poor survival. High expression of ALDH1 in Stage I have been associated with higher tumour recurrence. In another study done on rats, it was seen that increased ALDH1A1 expression was associated with poor survival in a cohort of NSCLC patients.[21] In this study, there were 29 cases (Stage 1–5, Stage 2–8, and Stage 3–16 cases) showing positivity however lack of follow-up is a major limitation of this study and hence there is a need of larger sample size studies with follow up to be performed. Some preliminary studies have reported poorer prognosis and survival of patients with higher expression of ALDH1[26] as seen in our cases where all the cases in Stage IV were positive for the marker. Variation in expression of these isoforms specially when using a polyclonal antibody for testing should be kept in mind while interpreting results. However many studies have shown that ALDH1A1 has poorer prognostic value especially in breast, ovary and lung patients.[34,35] Hou *et al.*[36] have shown that ALDH1 is associated with poorer patient prognosis and higher staging. In a study the expression of ALDH1A1 was positively correlated with the stage and grade of lung tumors and related to a poor prognosis for patients with early-stage lung cancer, which suggested that ALDH1A1 could be a potential prognostic factor and therapeutic target for the treatment of patients with lung cancer. Wang *et al.* and Li *et al.* saw that, higher ALDH1A1 expression levels were associated with a higher stage of disease (Stage III + IV) and poor survival[28,37] Jiang *et al.* showed that the ALDH1A1-positive lung cancer cells could generate tumors *in vivo*.[21] However, Dimou *et al.*[38] reported contradictory results, indicating that ALDH1A1-negative expression in lung cancer patients corresponded to shorter survival compared with those with ALDH1A1-positive expression and that ALDH1A1 over expression was associated with a favorable outcome. Some studies have reported the opposite of what is observed in present study, i.e., low levels of marker correlating with poor outcome. This could be attributed to various isoforms of ALDH1 existing (ALDH1A1, A2, A3, B1, L1, and L2).

CONCLUSION

Strong ALDH1 expression correlates with higher stage of lung carcinoma making it a prognostic marker needing in-depth study. However, identification and analysis of ALDH1 as a CSC can open doors in the field of cancer chemotherapeutics and prognosis.

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There are no conflicts of interest.

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