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Oncogene Expressions Detected by *in situ* Hybridization of Squamous Metaplasia, Dysplasia and Primary Lung Cancer in Human*

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In order to elucidate the dynamic changes of oncogene expression in the sequential cascade of squamous metaplasia, dysplasia, and squamous cell carcinoma of the bronchial epithelium, hybridization in situ was employed with a biotinylated oncogene probe. The expression of c-myc was localized exclusively in nuclei. While normal bronchial epithelium revealed no discernible clumps of c-myc grains, except occasional grains less than 3 per cell, squamous metaplasia showed increased number of grains and a few clusters of c-myc grains. In dysplasia, c-myc expression was more intensive than in squamous metaplasia. Approximately, 1/3 to 2/3 of tumor cell populations of squamous cell carcinomas of the lung revealed tremendously increased c-myc expression. In addition clumpy grains of c-myc in squamous cell carcinoma appeared more frequently than in squamous metaplasia or dysplasia. The c-myc expression was found to vary between different samples and within each cancer, and not all cancer cells expressed c-myc. These data indicate that c-myc oncogene plays it's role on reprogramming for growth control of cell populations particularly in multistage carcinogenesis and progression of lung cancer. These dynamic alterations of c-myc expression suggest that neoplastic transformation may occur conceivably at the dysplastic phase eventually resulting in carcinoma in situ. This means, in turn, squamous dysplasia is a putative precancerous lesion of the human lung.

Key Words: *C-myc Oncogene, In situ hybridization, Bronchial squamous metaplasia, Dysplasia, Squamous cell carcinoma*

INTRODUCTION

A number of approaches are generally employed in study of oncogenes in human neoplasia. In the first approach, human tumor cell DNA is mapped by restriction enzyme analysis in order to investigate polymorphic changes in human genomic homologs of oncogenic retroviruses originally detected in avian and rodent systems (Pulciani et al., 1982; Shimizu et al., 1983; Kan, 1987; Santos et al., 1984). In the next approach, oncogene encoded mRNA or protein product is analyzed by northern or western blotting (Griffin et al., 1985; Tanaka et al., 1986; Pulciani et al., 1982). The third approach refers to transfection of oncogenes from human tumors into the mouse NIH 3T3 fibroblastic cell lines ensuing a sub-

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sequent isolation of the transforming DNA genom (Der et al., 1982; Yoakum et al., 1985; Gemma et al., 1988). The fourth avenue of approaches permits us to elucidate directly the oncogene expression in either target tissue cells (Lee et al., 1988; Grady-Leopardi et al., 1986; Lee and Lee., 1988; Schmid et al., 1989) or target chromosome (Alitalo et al., 1983), usually termed "in situ hybridization." Thus in situ hybridization is now widely applied to estimate the cellular distributions of oncogene expressions and to detect target DNA or RNA in tissue sections of the hosts infected by various causative organisms (Lee and Lee, 1989; Lacy J et al., 1986; Nagy et al., 1988; Lee and Lee, 1988; McDougall et al., 1982; Ostrow et al., 1987; Niedobitek et al., 1989; Wolber et al., 1989; Donovan et al., 1988; Haase et al., 1985).

Some oncogenes are thought to play their roles in the multistage carcinogenesis and progression of neoplasms (Land et al., 1983). Alterations of oncogenes such as point mutation (Capon et al., 1983; Reddy et al., 1982; Cohen and Levinson, 1988), rearrangement (Dalla-Favera et al., 1982; Neel et al., 1982; Taub et al., 1982), amplification (Little et al., 1983; Nau et al., 1984) and overexpression (Seeger et al., 1985) are recognized in the various human cancers including lung cancers.

C-myc is the cellular homologue of the avian myelocytomatosis virus oncogene. Substantially, c-myc oncogene product binds DNA and seems to confer regulating the cellular proliferation (Rabbitts et al., 1985; Studzinski et al., 1986; Persson and Leder, 1984). Hence immortalization of cancer cells may involve alteration of c-myc oncogene. In view of elevated expression of c-myc during persistent rounds of cell cycle providing a reprogramming of growth control and an opportunity of incidental mutation, it is reasonable to suppose that atypical bronchial epithelium is a potential precancerous lesion eventually resulting in lung cancer as similar alterations in gastric mucosa do to stomach cancer (Saccomanno et al., 1974; Berkheiser, 1965; Lee and Lee, 1989; Saraga et al., 1987; Morson et al., 1980).

Present study is aimed at a goal figuring the dynamic changes of c-myc expression among squamous metaplasia, dysplasia, and squamous cell carcinoma of bronchial epithelium employing a highly sensitive technique of hybridization in situ.

MATERIALS AND METHODS

Human Tissue Samples

Formalin-fixed and paraffin-embedded tissue specimens of the bronchial mucosa of 19 patients undertaken by bronchofiberscopic biopsy were investigated in this study. The biopsy specimens in a patient were undertaken at least out of three different foci. Histopathologic diagnoses consisted of six squamous cell carcinomas, five dysplasias, and four squamous metaplasias of bronchial epithelia and four normal bronchi as a control.

Diagnostic criteria of the dysplasia and squamous metaplasia were adapted from the other investigators' work (Saccomanno et al., 1974). The dysplasia of the brochial epithelium represented the moderate atypia cited in the previous article.

Pretreatment of Microscopic Slides

Microscopic slides were pretreated as in our previous studies (Lee et al., 1988; Lee and Lee, 1988; Lee and Lee, 1988; Lee and Lee, 1989). Coating with Denhardt's medium and acetylation were carried out according to the method of Haase et al (1984). The pretreated microscopic slides were stored at room temperature until use.

Hybridization Probe

C-myc cDNA was produced from a 9kb Eco RI-Hind III fragment originating from a human neuroendocrine tumor cell line (COLO 320 HSR) (Alitalo et al., 1983) cloned into pBR322. The c-myc oncogene was nick translated by the method of Rigby et al. (1977) using a biotinylated-11-dUTP by Enzo Bio-chem (New York, NY).

In Situ Hybridization

In situ hybridization was performed as in our previous studies (Lee et al., 1988; Lee and Lee, 1988; Lee and Lee, 1989). Briefly, paraffin sections were cut onto pretreated clean slides. Dewaxed and rehydrated sections were fixed in Carnoy's A solution, dipped in 0.1% Triton X-100 in phosphate buffered saline (PBS), treated with 0.2N HCl for 20 minutes to facilitate the penetration of the probe by diffusion, rinsed in 2X SSC (0.3M soium chloride/0.03M sodium citrate) and washed in double distilled water (DDW). Slides were then incubated in a solution containing 0.01mg/ml proteinase K (Sigma, St. Louis, MO), 20mM Tris HCl pH7.4 and 2mM $CaCl_2$ for 15 minutes at 37°C, followed by extensive washing in PBS containing 0.2% glycin (Sigma, St. Louis, MO), and submerged in PBS containing 4% paraformaldehyde (Sigma, St. Louis, MO) at room temperature for 20 minutes. After 3 successive 5-minute washings in PBS, the slides were agitated in 95% deionized formamide in 0.1X SSC solution at

65°C and dipped in a mixture of ice and 0.2X SSC. After sequential dehydration through graded ethanol the slides were then ready for prehybridization.

Prehybridization mixture consisted of 50% deionized formamide, 3X SSC 10% dextran sulfate (Phamacia, Uppsala, Sweden), 200μg/ml sperm DNA (Enzo Biochem, New York, NY), 100μg/ml polyadenylate (Sigma, St. Louis, MO), 0.2% bovine serum albumin (BSA), 0.02% ficoll, and 0.02% polyvinylpyrolidone. Approximately 25μl of hybridization mixture was applied to a section. Sections were then covered with sterile clean glasses, and incubated at 37°C for one hour. After applying 10μl of a hybridization solution contained 20μg/ml biotinylated c-myc cDNA probe in a prehybridization mixture on a tissue section and covering with sterile, pretreated coverslips, both of target cellular DNA and probe cDNA were denatured together by placing the slides at 90°C for 3 minutes in a humid chamber. Hybridization reaction was performed overnight at room temperature and then rinsed sequentially for 5 minutes each in 2X SSC, 50% deionized formamide/0.1X PBS, and 0.05% Triton X-100 in PBS.

Detection of Hybridization Signals

After rehydrating for 20 minutes in 1X TTBS (0.1M Tris HCl pH7.5, 0.1M NcCl, 2mM MgCl2, 0.05% Triton X-100, 3% crystalline grade BSA), 100μl of Vectastain ABC-AP reagent (Vector Lab, Burlingame, CA) was flooded on each section of slides. And then color reaction was developed with alkaline phosphatase substrates mixture of nitro blue tertazolium/bromochloroindolylphosphate (NBT/BCIP). The slides were counterstained with hematoxylin and eosin (H & E) and washed in DDW.

Specificity Tests for Hybridization in Situ

A couple of controls were used to evaluate the specificity of the hybridization of the biotinylated c-myc cDNA probe to the tissue samples. These were in situ hybridization without the probe cDNA, and in situ hybridization with probe after treating the tissue sections with pancreatic deoxyribonuclease (DNAse) (Sigma, St. Louis, MO) and ribonuclease T1 (RNAse) (Sigma, St. Louis, MO) before the prehybridization step.

RESULTS

The expressions of c-myc oncogene were localized exlusively in nuclei. Most of the normal bronchial epithelia revealed no discernible clumps of c-myc grains that consisted of 5 or more grains per cell. C-myc grains, however, occasionally were less than 3 per cell in the normal bronchial epithelia (Fig. 1 & 2). By contrast, squamous metaplasia showed several cells expressed c-myc, the grains of which were 5 to 10 per cell and only a few clusters of c-myc grains within a microscopic field (Fig. 3). Nuclear clusters of c-myc grains were more intensive in dysplastic lesions that showed occasional cells expressed c-myc grains of 5 to 15 per cell (Fig. 4-6). C-myc positive cells were usually less than 10% of cell populations in squamous metaplasia or dysplasia.

Approximately, 1/3 to 2/3 of populations of each squamous cell carcinoma of lung represented the enhanced c-myc expression. Number of grains per

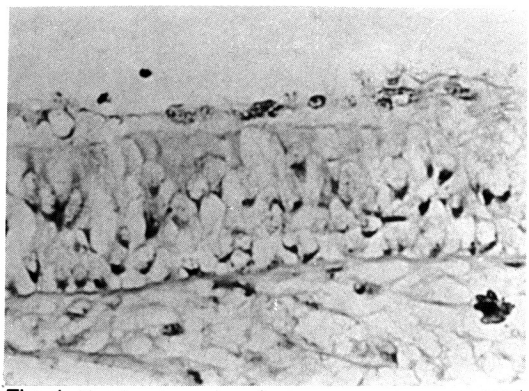

Fig. 1. No clumpy grains of c-myc oncogene expressed in a normal bronchial mucosa. In situ hybridization using biotinylated c-myc probe to formalin-fixed, paraffin-embedded tissue section where immunohistochemical reaction of ABC-AP with substrate mixture of NBT and BCIP was employed to detect target signals (counterstained with H & E, 400×).

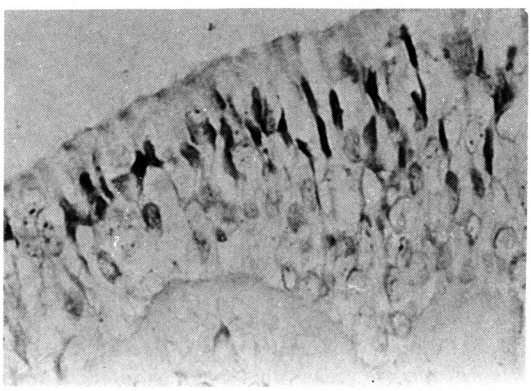

Fig. 2. Nuclear c-myc grains were exclusively less than 3 per cell in a normal bronchial mucosa of the other subject (counterstained with H & E, 400×).

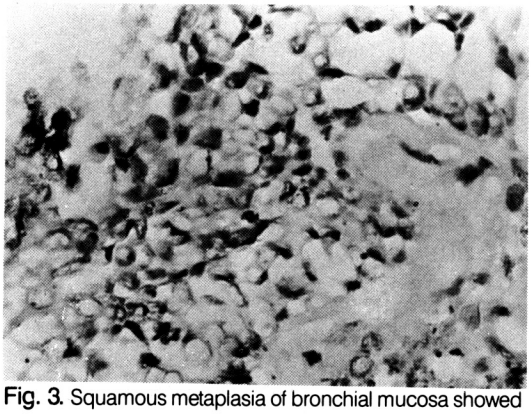

Fig. 3. Squamous metaplasia of bronchial mucosa showed several cells expressed c-myc grains of which were 5 to 10 per cell, and only a few clusters of c-myc grains (counterstained with H & E, 400×).

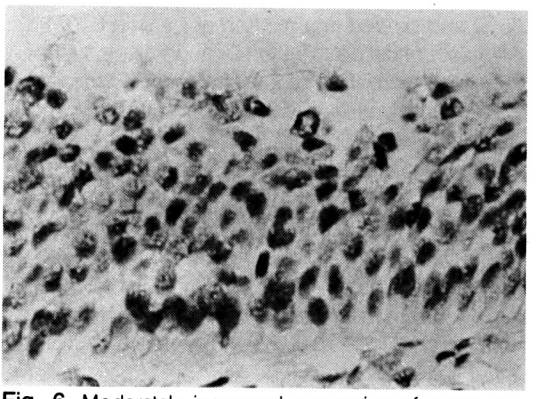

Fig. 6. Moderately increased expression of c-myc oncogene in a dysplasia of bronchial mucosa in the other patient (counterstained with H & E, 400×).

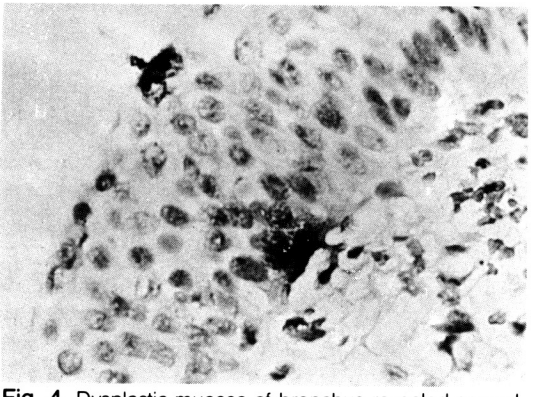

Fig. 4. Dysplastic mucosa of bronchus revealed several nuclei indicating clumpy grains of the intensive c-myc expression (counterstained with H & E, 400×).

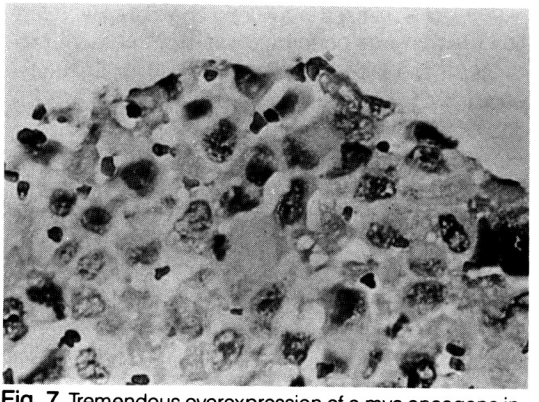

Fig. 7. Tremendous overexpression of c-myc oncogene in a squamous cell carcinoma of the lung. Cell-to-cell variation in intensity of c-myc expression can be assessed, and not all tumor cells expressed c-myc (counterstained with H & E, 400×).

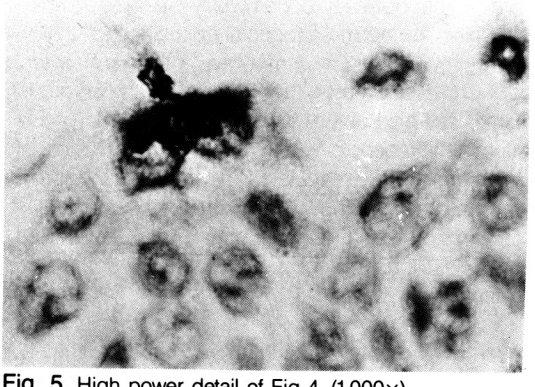

Fig. 5. High power detail of Fig 4. (1,000×).

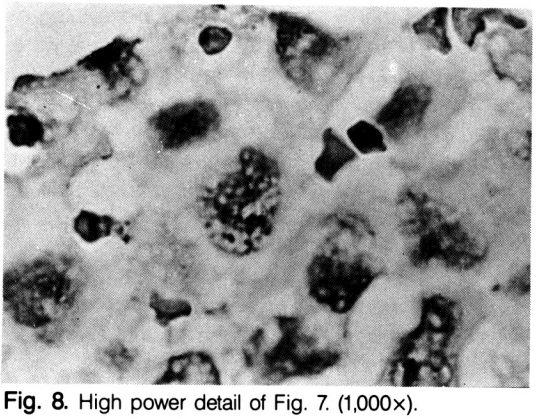

Fig. 8. High power detail of Fig. 7. (1,000×).

nucleus of cancer cells were estimated as 5 to 60, and clumpy grains in squamous cell carcinoma appeared more frequently than in squamous metaplasia or dysplasia (Fig 7, 8). The expression of c-myc was found to vary between different samples and within each tumor, and not all cancer cells expressed c-myc. Cell-to-cell variation in intensity of c-myc enhancement was also assessed.

For specificity testing, pretreating with DNAse and RNAse completely abolished labeling with the biotinylated c-myc cDNA to the target tissue sections. Also, no signal was detectable by employing hybridization in situ with exclusion of c-myc probe.

DISCUSSION

Obviously, a variety of hyperplastic, atypical, and dysplatic changes can be found in the epithelium of conducting air ways probably, related to smoking and environmental pollutants. Although the true significance of those lesions is so far unclear, it is likely that most such changes are reactive rather than neoplastic. Nontheless, serial cytologic studies have demonstrated that squamous cell carcinoma of the lung appears to arise from a sequence of metaplastic and dysplastic cells, eventuating first in squamous carcinoma in situ and then in invasive carcinoma (Saccomanno et al., 1974; Berkheiser, 1965). Therefore, dysplasia is thought to be a dynamic process capable of regression or progression. Despite current accumulation of knowledge about oncogene activation in human lung cancer, the expression of oncogene has not been definitely elucidated on that aspect.

In the present study, all cases of squamous cell carcinoma of the lung examined revealed heterogeneous augmentation of c-myc expression. The intensity of c-myc expression, localized in cell nucleus that is consistent with the other reports (Lee and Lee, 1989; Persson et al., 1984) was strong in squamous cell carcinoma, whereas that was relatively weak in squamous metaplasia and dysplasia of the bonchial epithelium. Moreover, dysplasia showed more intensive enhancement of c-myc oncogene expression than squamous metaplasia. These dynamic changes of c-myc expression suggest that neoplastic transformation may occur particularly in dysplastic phase of the sequential cascade of the development of squamous cell carcinoma of the lung. And also, our findings are consistent with that c-myc plays it's role on reprogramming for growth control of cell populations particularly in multi-stage carcinogenesis and progression of cancer (Land et al., 1983; Lee et al., 1988; Lee and Lee, 1988; Lee and Lee, 1989).

In view of a point that regulation of cellular proliferation is a complex process that involves both positively and negatively acting signals, carcinogenesis may result from alterations in genes of which protein products are invo lved in these signal pathways. In this regard, a set of proteins that are probably capable of reprogramming normal regulation of cellular growth for immortalization possibly collaborate with other oncogene. By contrast, the oncosuppressors or anti-oncogenes, represented in retinoblastoma, have also been implicated in carcinogenesis but obviously behave in a different way, in which cancer formation ensues when the loss or inactivation of a single copy of the putative oncosuppressor gene appears (Ponder, 1988; Green, 1988; Spandidos and Anderson, 1989). This suggests that anti-oncogenes normally act in holding cellular proliferation. Association of oncogenes and anti-oncogenes may appear as constituents of a common regulatory pathway (Whyte, et al., 1988). So not all 'dominant' activated oncogenes are completely dominant, and not all loss of function mutations in oncosuppressor genes are truely recessive as Ponder (1988) has indicated already. Perhaps, this may be the reason why c-myc expression in human lung cancer appeared to vary between cell-to-cell even within the same sample, and why not all cancer cells expressed c-myc.

Alternatively, modulation of transcriptional elongation through specific DNA sequences within the c-myc gene may also provide an attractive mechanism for regulating its heterogeneously attenuated expression in cancers (Wright and Bishop, 1989).

On the other hand, the relationship of c-myc expression during transition of cells from Go to G_1 phase of cell cycle and essentially asynchronous nature of growth transitions in cancer cell populations seems to play a possible role in heterogeneous enhancement of c-myc expression during multi-stage carcinogenesis and progression of cancer cells.

Additionally, on the basis of new convincing evidence linking the oncogenes and growth factors, many, if not all, of the oncogene products may be involved in the growth factor-receptor-response pathway and this indicates the points at which alterations may occur leading to the development of a neoplastic transformation (Goustin et al., 1986). Constitutive activation of growth factor-regulated genes such as c-myc in certain circumstances of activation or inappropriate expression results in an apparently continuous stimulus to drive proliferation. The precise reason, however, behind the heterogeneous expression is not

yet understood.

In conclusion, dynamic changes of c-myc enhancement are involved during the sequential cascade of squamous metaplasia-dysplasia-squamous cell carcinoma. And this, in turn, strongly supports that dysplasia is a potential precancerous lesion of squamous cell carcinoma of the lung.

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