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Genetische Heterogenität in Adenokarzinomen des Ösophagus (Barrettkarzinom)

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GENETIC HETEROGENEITY IN ESOPHAGEAL (BARRETT'S) ADENOCARCINOMA

Abstract

Genetische Heterogenität in Adenokarzinomen des Ösophagus (Barrettkarzinom)

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Die intratumorale genetische Heterogenität ist ein bislang wenig untersuchter Aspekt der molekularen Karzinogenese, der potentiell eine erhebliche tumorbiologische und therapeutische Bedeutung aufweist. In der vorliegenden Arbeit wurde die genetische Heterogenität beim Adenokarzinom des Ösophagus systematisch untersucht. Grundlage waren 10 Patienten mit einem Adenokarzinom des Ösophagus, die sich einer Resektionsbehandlung ohne vorangegangene Radio- oder Chemotherapie unterzogen hatten. Nach Formalinfixation der Operationspräparate wurden aus den vollständig in Paraffin eingebetteten Primärtumoren jeweils acht repräsentative Areale (aus Tumorzentrum und -peripherie jeweils an der Tumoroberfläche und der Invasionsfront), eine regionäre Lymphknotenmetastase sowie tumorfreies Normalgewebe für die nachfolgenden molekularbiologischen Untersuchungen ausgewählt. Nach DNA-Präparation aus mikrodisseziertem Gewebe wurden Fragmente der Tumorsuppressorgenloci APC, MCC, RB, D4S1652 und D18S474 sowie der Protoonkogenloci c-erbB-2, c-myc und EGFR mit Hilfe der PCR amplifiziert. Anschließend wurden die PCR-Produkte aus den verschiedenen Tumorarealen anhand von Silber-gefärbten Polyacrylamidgelen auf einen Allelverlust (LOH) in den 5 Tumorsuppressorgenloci hin geprüft. In den 3 untersuchten Protoonkogenloci erfolgte der Nachweis einer Genamplifikation mit Hilfe einer differentiellen PCR, die neben dem untersuchten Zielgen ein nicht amplifiziertes Kontrollgen einschließt.

Dabei ergab sich ein LOH im APC-Gen in 11% der informativen Fälle, im MCC-Gen in 57%, im RB-Gen in 50%, im Locus D4S1652 in 75% und im Locus D18S474 in 0% der Fälle. Genamplifikationen fanden sich am häufigsten im c-myc-Gen (33%), während eine Amplifikation in den Genen c-erbB-2 und EGFR mit jeweils 10% deutlich seltener nachweisbar war. Hinsichtlich der intratumoralen genetischen Heterogenität konnte gezeigt werden, daß in 94% der Fälle mit nachgewiesener genetischer Aberration (LOH oder Genamplifikation), diese nur in einem Teil der Tumorareale vorhanden war, während die anderen Areale dem Wildtypstatus entsprachen. Bezüglich der topographischen Verteilung genetischer Aberrationen ließen sich jedoch keine signifikanten Unterschiede zwischen Tumorzentrum und -peripherie bzw. zwischen Tumoroberfläche und Invasionsfront nachweisen. Die Ergebnisse zeigen deutlich, daß es sich bei der intratumoralen genetischen Heterogenität beim Adenokarzinom des Ösophagus um einen häufigen Befund handelt, der bei künftigen Untersuchungen molekularbiologischer Veränderungen bei diesem Tumortyp in Betracht gezogen werden muß.

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Datum:.....

1. Literature Review

1.1 Introduction

There has been a remarkable increase in incidence of esophageal adenocarcinoma arising in association with, and as a consequence of a metaplastic columnar transformation of the normal squamous lining epithelium of the esophagus, so called Barrett's esophagus. This increase is particularly noticeable in the countries of Western Europe and North America (Devesa SS et al., 1998; Pera M et al., 1993; Blot WJ et al., 1991; Reed PI, 1991; Powell J & McConkey CC, 1990; Hesketh PJ et al., 1989; Cameron AJ et al., 1985).

In these countries, Barrett's adenocarcinoma is very common especially among middle-aged white males in whom a 350% increase in incidence was recorded between the mid 1970s and the early 1990s (Devesa SS et al., 1998). Beginning with occasional single case reports in the 60s and 70s (Haggitt RC et al., 1978; Stillman AE & Selwyn JI., 1975), with only 32 reported cases of invasive adenocarcinoma of the esophagus in the literature as at 1978 (Berenson MM et al., 1978), it is now one of the 15 most common cancers in the United States of America (Blot WJ et al., 1991). There, it recorded the highest rate of increase in incidence among all cancers between the 70s and the 90s, surpassing even the rates for non-Hodgkin lymphoma, skin melanoma and lung cancer (Devesa SS et al., 1998). Barrett's esophagus is an acquired condition, which occurs as a complication in about 8-20% of cases of symptomatic reflux esophagitis (Haggitt RC, 1992; Spechler SJ & Goyal RK, 1986; Dahms BB & Rothstein FC, 1984; Hamilton SR & Yardley JH, 1976; Mossberg SM, 1966). It has been demonstrated to be a predisposing condition to cancer (Berenson MM et al., 1978) by progressing from metaplasia through dysplasia to adenocarcinoma (Hamilton SR & Smith RRL, 1987; Menke-Pluymers MBE et al., 1993; Gleeson CM et al., 1998). In spite of the opportunity for early detection and treatment provided by the observed metaplasia-dysplasia-carcinoma sequence, the prognosis in Barrett's adenocarcinoma remains very poor (Fein R et al., 1995) due to several factors. Survival rate in Barrett's adenocarcinoma is strongly correlated with stage on presentation (Streitz JM et al., 1991) but a large proportion of the patients present with advanced disease (Fein R et al., 1995) so that relatively little experience has been gained in the management of the early stages (Rusch VW et al., 1994; Rice WR et al., 1993). There is also a great interobserver variability in the histological diagnosis of the early preinvasive stages (Reid BJ et al., 1988a) and their biological behavior remains poorly elucidated so that their best mode of management is a source of great controversy (Pera M et al., 1992; Levine DS et al., 1993).

For these reasons, there is currently great interest in gaining a better understanding of the pathogenesis of Barrett's adenocarcinoma and its preinvasive dysplastic lesions. Several studies have been designed to gain a better insight into this field, ranging from endoscopic surveillance of symptomatic Barrett's esophagus patients, through histologic and immunohistochemical characterization of the different stages of neoplastic progression, to analysis of subcellular genetic and molecular abnormalities and their correlation with biologic behavior. In spite of much efforts, especially at the genetic level, a characteristic molecular aberration or a definite pattern of acquisition of genetic abnormalities, which could serve as a reliable basis for therapeutic decision making is yet to be found. This difficulty has been ascribed to various factors like differing methods of investigations from one center to the other and the relatively few number of cases available to individual investigating group (Fitzgerald RC & Triadiafilopolous G, 1998). However, a potential confounding factor is the possibility of intratumoral genetic heterogeneity. This is a phenomenon that has been demonstrated in several human cancers (Macintosh CA et al., 1998; Diaz-Cano SJ et al., 2000; Lichy JH et al., 2000) and has been shown to be capable of impeding the detection of genomic abnormalities in breast carcinoma (Aubele M et al., 1999). There are very limited studies on intratumoral heterogeneity in esophageal cancers (Haraguchi Y et al., 1995; Robaszkiewicz M et al., 1992; Sasaki K et al., 1991) from a very few number of authors who employed flow cytometry to analyze DNA content from different regions of a tumor. Flow cytometry is however, a very unspecific method that cannot provide any information at the highly important genetic level of carcinogensis. Nevertheless, there is to date, no study of intratumoral heterogeneity in Barrett's adenocarcinoma at the genetic level. Thus, this work represents the first attempt at investigating this highly important process with enormous potential influence on Barrett's cancer research and therapy.

1.2 Barrett's Esophagus (BE)

This is the metaplastic transformation of the normal squamous epithelial lining of the distal esophagus, above the gastroesophageal junction (GEJ), into a columnar-type epithelium resembling that of the stomach or intestine (Spechler SJ & Goyal RK, 1996; Hamilton SR & Yardley JH, 1977; Mossberg SM, 1966). The condition was first described in 1906 by Tileston (Tileston W, 1906) and about 30 years later by Lyall (Lyall A, 1937). It was, however, the description in 1950 by Norman Barrett of a group of patients with midesophageal stricture and ulceration, in which the distal esophagi were lined by columnar epithelium, that popularized the

condition (Barrett N, 1950), hence the widely used eponym of Barrett's esophagus (BE) for this condition. Similar histologic changes have since been reported by many other authors (Hamilton SR & Yardley JH, 1977; Mossberg SM, 1966; Paull A et al., 1976) who, however, differed from the original proposition of Barrett that the condition resulted from a congenitally short esophagus with accompanying intrathoracic displacement of the stomach. Although there is evidence for congenital rests of gastric-type columnar epithelium in the esophagus of normal individuals (Spechler SJ & Goyal RK, 1986), the acquired nature of the condition is widely accepted and has been demonstrated in several clinical and experimental studies (Mossberg SM, 1966; Bremner et al., 1970; Hamilton SR & Yardley JH, 1977).

1.2.1 Epidemiology

Occurrence of BE is frequently reported in middle-aged patients (Paull G & Yardley JH, 1988; Brand LD et al., 1980) but has also been reported in children (Dahms BB & Rothstein FC, 1984; Hassal H et al., 1985; Dahms BB et al., 1987) in whom a frequent association with mental retardation was found (Qualman JS et al., 1990). BE arises usually but not exclusively as a complication of chronic gastro-esophageal-reflux (GER), (Dahms BB & Rothstein FC, 1984; Hassal H et al., 1985; Hamilton SR & Yardley JH, 1977) with a prevalence of about 8-20% in patients with symptomatic esophagitis undergoing endoscopy (Sarr MG et al., 1985; Spechler SJ & Goyal RK, 1986). Other reported predisposing factors include cytotoxic drug therapy (Dahms BB et al., 1987) and familial predisposition (Fahmy N & King JF, 1993). White race, alcohol and smoking have also been suggested as possible predisposing factors (Spechler SJ & Goyal RK, 1986) whereas infectious agents like Helicobacter pylori still remain to be completely ruled out (Jankowski JA et al., 1999; Paul G & Yardley JH, 1988).

1.2.2 Pathogenesis

The pathogenesis of BE is still poorly understood, but it is proposed to involve inflammation of the normal squamous epithelium due to recurrent gastro-esophageal-reflux (GER). Initially, this leads to adaptive changes of esophagitis i.e. acanthosis and papillomatosis, which results in the functional stem cells at the tip of the papillae coming into a relatively superficial position where they are more accessible and susceptible to refluxed or ingested chemicals (Jankowski J, 1993).

In 8-20% of patients, the GER is persistent and leads to ulceration (Jankowski JA et al. 1999). Re-epithelialisation occurs presumably by multipotent undifferentiated stem cells, which in the milieu of acid, pepsin and probably bile, differentiate into the various types of columnar epithelial cells characteristic of BE (Spechler SJ & Goyal RK, 1986). The primal origin of the undifferentiated stem cell remains unclear though. Postulates include origin from heterotopic rests of columnar epithelium known to occur in normal individuals (Paull A et al., 1976), in-growth of undifferentiated neck cells of normal gastric fundic glands (Hamilton SR & Yardley JH, 1977), altered differentiation of stem cells of squamous mucosa or associated glandular ducts (Jankowski JA et al., 1999), and aberrant metaplastic differentiation of mesenchymal cells, which not only produce the columnar epithelium, but also stromal tissue and an extra layer of muscularis mucosa (Takubo K et al., 1991). Ultrastructural and molecular studies revealed great heterogeneity in the columnar cell population in BE (Kumble S et al., 1996) with remarkable similarity between the mucous granules in the BE columnar epithelium and gastric mucous neck cells (Levine DS et al., 1989) but a definite origin from these cells has not been established. The development of the specialized epithelium in BE is age-related (Qualman SJ et al., 1990; Hassal E at al., 1985) and does not regress even after anti-reflux therapy (Naef APJ et al., 1975; Hamilton SR et al., 1984; Qualman SJ et al., 1990; Jankowski JA et al., 1999). Occasional reports of regression in BE following surgical and medical therapy (Brandl DL et al., 1980; Hassal E at al., 1993) are possibly due to sampling errors (Spechler SJ & Goyal RK, 1986). Although photoablation therapy to remove the BE may allow re-epithelialisation with squamous epithelium, there is persistent underlying glandular epithelium, which can lead to recurrence (Haggitt RC, 1994) or even lead to the development of adenocarcinoma (Laethem JL et al., 2000). The inherent potential of BE to progress into dysplasia, a precursor for malignant disease, makes it a preneoplastic lesion (Chejfec G et al., 1992), and justi fies the level of attention it currently enjoys.

1.2.3 Diagnosis of BE

The clinical diagnosis of BE using non-invasive techniques like barium esophagogram is non-specific whereas the characteristic orange color and velvety appearance of BE on endoscopy made this method the mainstay of diagnosis (Petras RE et al. 1991). (see fig. 1 showing endoscopic appearance of BE). The determination of anatomical landmarks for defining GEJ on endoscopy, however, are either non-specific or clinically inapplicable (Spechler SJ & Goyal RK, 1986), hence,

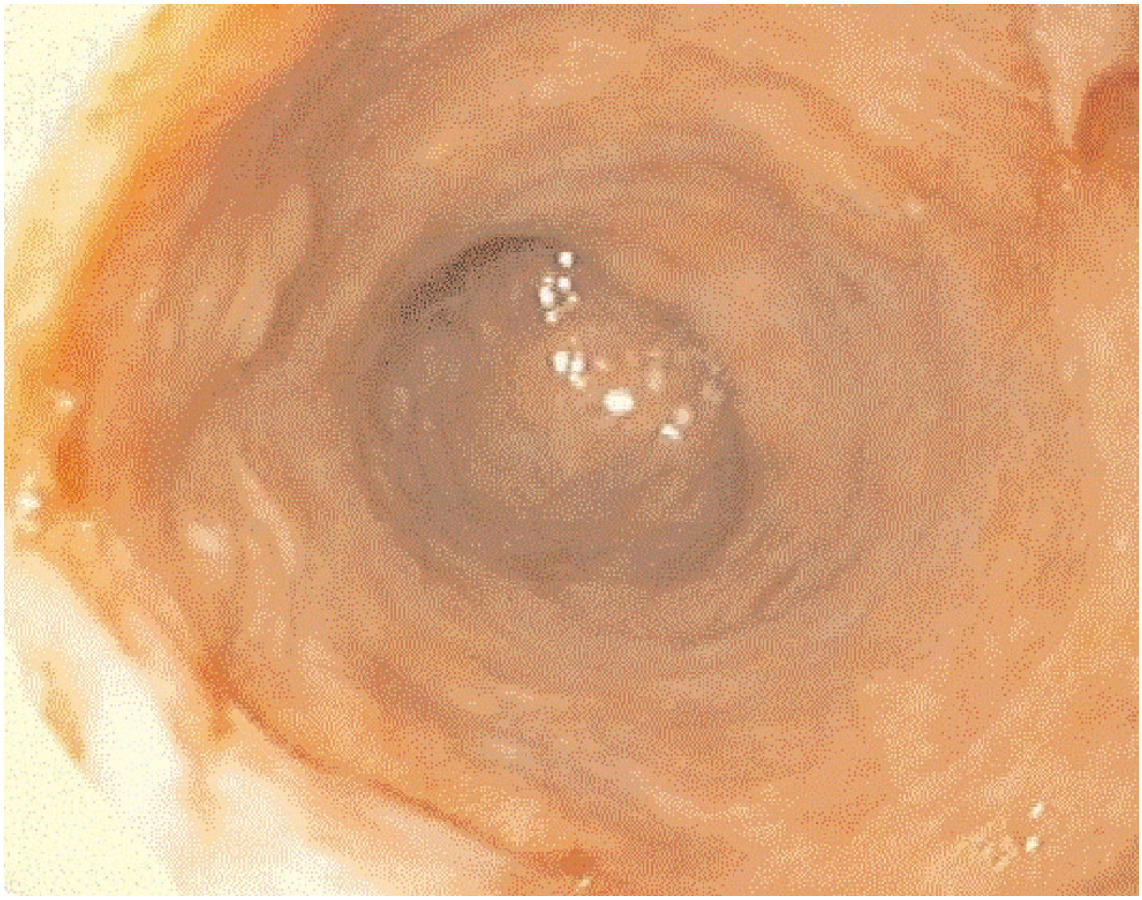


Fig. 1 Endoscopic appearance of Barrett's esophagus showing velvety orange color appearance compared with the whitish appearance of normal esophageal mucosa.

histologic confirmation of suspected BE through an endoscopic biopsy from the site is necessary for diagnosis. Three types of metaplastic columnar epithelium characterized BE originally. These were gastric-fundic, junctional or cardiac and intestinal or specialized epithelial types (Brand DL et al., 1980; Paull A et al., 1976; Dahms BB & Rothstein FC, 1984). There is great similarity between the columnar surface epithelium in BE and normal foveolar or intestinal absorptive cells on routine histology, and a reliable differentiation is possible only through electron microscopy and histochemistry (Levine DS et al., 1989; Haggitt RC, 1994). Furthermore, endoscopic determination of GEJ using the squamo-columnar junction is not always accurate since this does not exactly correspond to the lower esophageal sphincter (LES) and there is a frequent occurrence of cardiac and gastric-fundic epithelium within the LES in normal individuals (Gottfried MR et al., 1989; Hassal E at al., 1985). Also, hiatal hernia is frequently associated with BE (Sarr MG et al., 1985) and may be mistakenly biopsied (Hassal E at al., 1985). For these reasons, an acceptable histologic diagnosis of BE is currently based on the demonstration of distinctive specialized intestinal metaplasia (Haggitt CR, 1994; Gottfried MR et al., 1989; Hassal E et al., 1985), with or without villar architecture (Thompson JJ, 1983). (fig. 2)

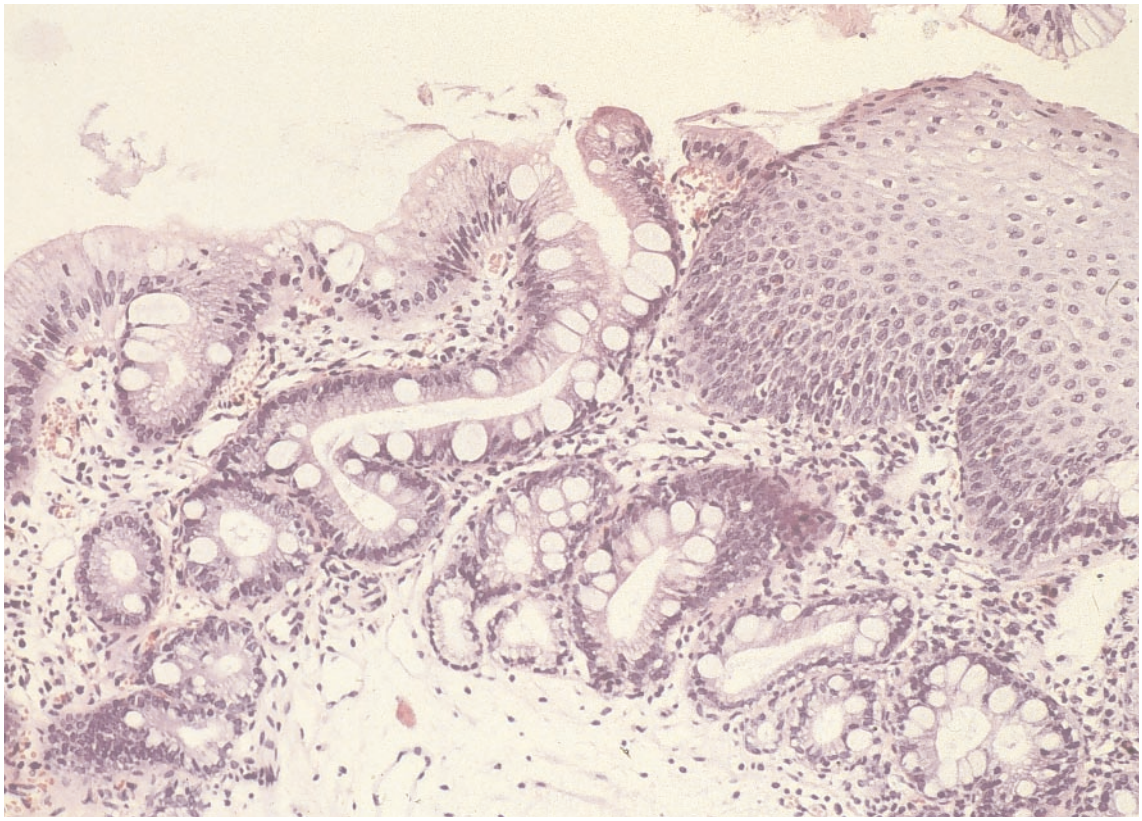


Fig. 2 Histologic appearance of Barrett's epithelium showing distinctive specialized large intestinal type epithelium with characteristic Goblet cells. Note adjacent squamous epithelium of the esophagus.

1.3 Dysplasia in BE

The unequivocal presence of neoplastic alteration of the columnar mucosa in BE, which is still confined within the basement membrane, is referred to as dysplasia (Spechler SJ & Goyal RK, 1986). Dysplasia is characterized by a spectrum of cytologic and architectural abnormalities including crowded, stratified, hyperchromatic nuclei, sometimes with prominent nucleoli and mitosis. The glands are enlarged and distorted with villiform or back-to-back, cribriform configuration (Haggitt RC, 1994). Based on the extent and severity of these changes, dysplastic changes in BE have been classified into different grades of negative, indefinite for dysplasia, low grade dysplasia (LGD) and high grade dysplasia (HGD) which includes carcinoma-in-situ (Petras RE et al., 1991). (fig. 3 showing dysplastic BE).

Dysplasia has been reported in all the three types of BE but more common and more severe in specialized intestinal type (Hamilton SR & Smith RRL, 1987). Prevalence of dysplasia in BE without associated adenocarcinoma is estimated at about 5-10% (Paull A et al., 1976; Spechler SJ & Goyal RK, 1986). A more frequent association with adenocarcinoma has also been noted, ranging between 68 and 100% (Rosenberg JC et al., 1985; Spechler SJ & Goyal RK, 1986).

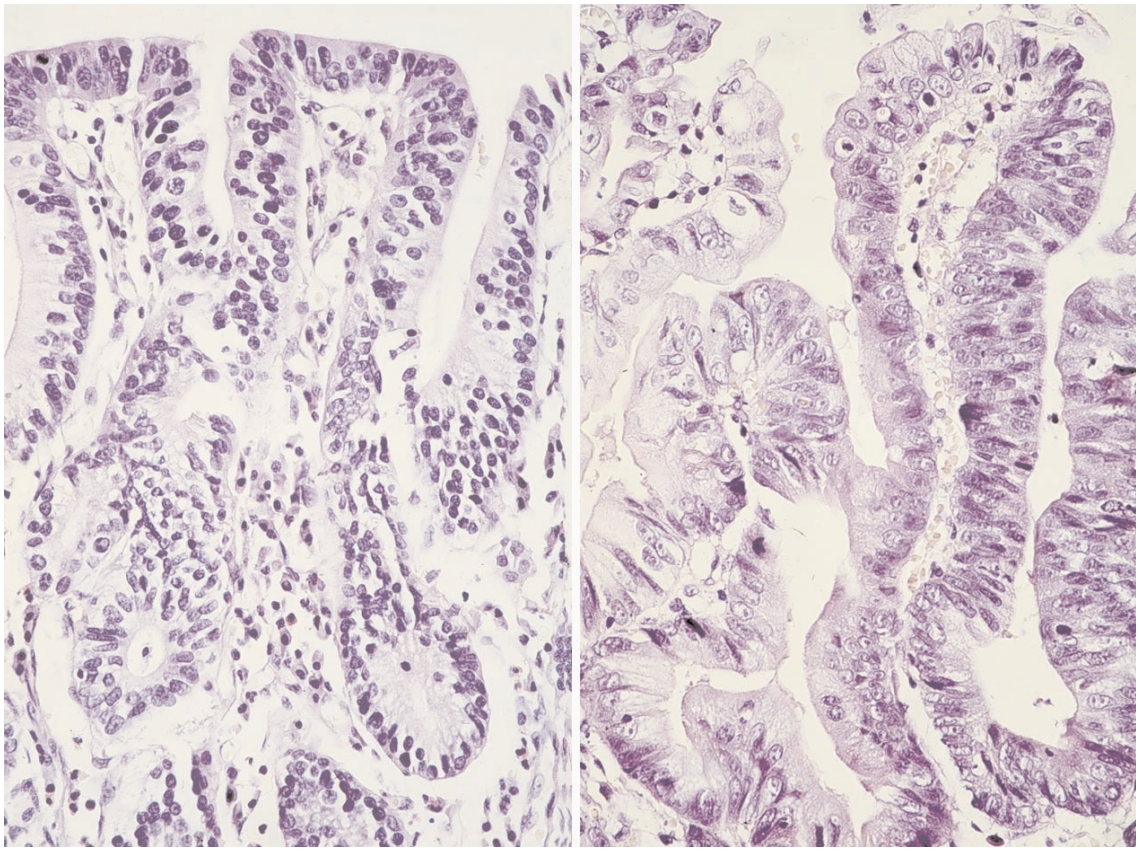


Fig. 3 Histologic appearance of dysplasia in Barrett's esophagus. Low grade dysplasia (left) and high grade dysplasia (right).

The presence of dysplasia in resected specimens of adenocarcinoma was initially regarded only as a marker for the presence of a carcinoma (Berenson MM et al., 1978). However, several carefully designed histologic studies supported the possibility of a causal relationship, leading to the recognition of dysplasia as a necessary precursor for adenocarcinoma in BE and the proposal for a dysplasia-adenocarcinoma sequence (Thompson JJ et al., 1978; Hamilton SR & Smith RRL, 1987; Haggitt RC et al., 1978). The proposed metaplasia-dysplasia-adenocarcinoma sequence has since been strengthened further by other histologic and flow cytometric studies (Gleeson CM et al., 1998; McArdle JE et al., 1992; Reid BJ et al., 1992).

1.4 Adenocarcinoma in BE

There is an increased risk of developing a primary esophageal adenocarcinoma in BE patients.(fig. 4)The simple presence of BE does not, however, lead to the development of adenocarcinoma, hence, majority of patients with simple BE do not develop invasive adenocarcinoma and they do not suffer any curtailment in longevity (Cameron AJ et al., 1985). Therefore, the occurrence of

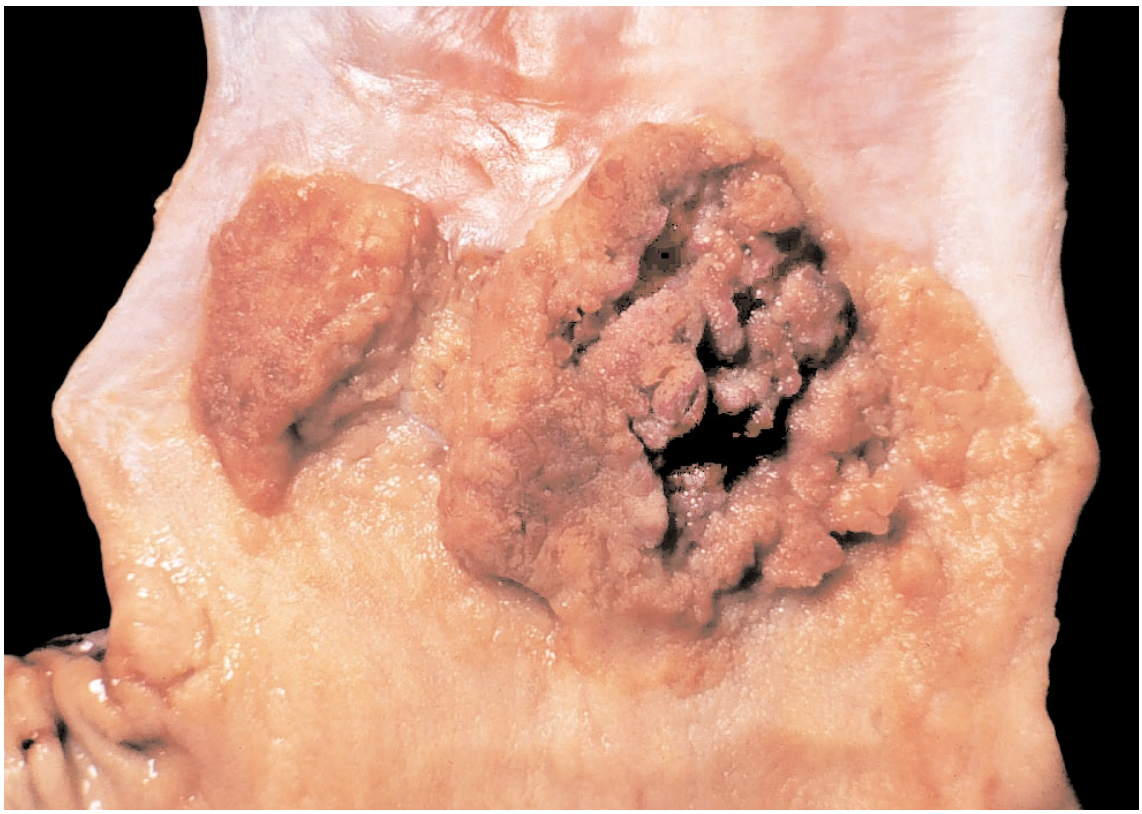


Fig. 4 Resected specimen of the esophagus showing ulcerated polypoid tumor mass located in an area of Barrett's epithelium.

adenocarcinoma in BE is believed to be influenced by other factors apart from the mere presence of BE.

The length of BE is an important risk factor (Spechler SJ & Goyal RK, 1986) with about 1.7 times increased risk of adenocarcinoma for doubling of any length of BE (Menke-Pluymers MBE et al., 1993). The development of adenocarcinoma in BE is also strongly associated with the presence of specialized epithelium and dysplasia especially HGD (McArdle JE et al., 1992). Persistent gastroduodenal refluxate, which has been demonstrated to be carcinogenic in laboratory animals (Taylor PR et al., 1991) has also been suggested, although effective anti-reflux surgery does not seem to prevent the subsequent development of invasive adenocarcinoma (Naef AP et al., 1975; Hamilton SR et al., 1984; Cameron AJ et al., 1985; Streitz JM et al., 1991). Other factors likely to contribute either directly or indirectly to progression to invasive cancer include, male sex, white race and life-style factors.

Concerning sex, Barrett's adenocarcinoma is more frequent in males (Sarr MG et al., 1985), much more frequent than could be explained by the observed male preponderance in benign BE (Rosenberg JC et al., 1985; Skinner et al., DB 1983). It shows the highest male to female ratio

after cancer of the lip (Blow WJ et al., 1991). A very recent experimental study using transgenic mouse models suggested that sex hormones and the presence of androgen receptors might play a determinant role in the frequent occurrence of esophageal adenocarcinomas in males (Tihan T et al., 2000).

With regards to race, reports of increasing incidence of Barrett's adenocarcinoma are from the regions of the world predominantly populated by Whites (Devesa SS et al., 1998; Hesketh PJ et al., 1989; Blot WJ et al., 1991). In multiracial communities of North America, the increased incidence is most appreciable in Whites (Sanfey H et al., 1985), in whom a 350% increase incidence was noted between the 1970s and the 1990s (Devesa SS et al., 1998). In fact, adenocarcinoma has become the predominant esophageal cancer in Whites compared with Blacks who have squamous cell carcinoma (SCC) predominantly (Blot WJ et al., 1991).

Life-style factors like smoking, alcohol and diet rich in nitrosamines but poor in fruits and vegetables as well as obesity, associated with the high socio-economic class are some of the factors that have been suggested by various authors (Powell J & McConkey CC, 1990; McDonald WC & MacDonald JB, 1987). Of all these factors, the strongest support obtains for smoking (Menke-Pluymers MBE et al., 1993) whereas large scale epidemiological studies would be needed to validate the individual contribution of the other factors (Fitzgerald RC & Triadafilopolous G, 1998). The initial assessments of risk of developing adenocarcinoma in BE were based on autopsy and hospital records rather than on longitudinal epidemiological studies (Petras RE et al., 1991). This led to exaggerated estimated relative risk values based on the reported incidence rates of about 10% (Naef AP et al., 1975; Spechler SJ & Goyal RK, 1986; Haggitt RC, 1994). These rates actually represented prevalence rates of adenocarcinoma in newly diagnosed BE cases, which vary between 7% and 16% (Miros M et al., 1991; Sarr MG et al.,1985; Cameron AJ et al., 1985; Spechler SJ et al., 1984). Subsequent follow-up studies of BE patients without adenocarcinoma on initial endoscopy gave incidence rates between 1 in 52 patient-years and 1 in 441 patient-years. This has been extrapolated by the authors as conferring between 30- and 125-folds increased risk above that of the general population (Miros M et al., 1991; Robertson CS et al., 1988; Cameron AJ et al., 1985; Spechler SJ et al., 1984; Hameeteman W et al., 1989). The real incidence of adenocarcinoma in BE is, however, difficult to assess because a considerable number of BE patients are asymptomatic (Petras RE et al., 1991). Nonetheless, since Barrett's adenocarcinoma is a relatively uncommon cancer in the general population, the absolute risk of developing the cancer in the general population is much lower than the high extrapolated risk (Spechler SJ et al., 1984).

1.5 Management and Prognosis

The risk for progression into invasive cancer is negligible in BE without dysplasia and quite low in the medium term in LGD (Miros M et al., 1991). Patients with these diagnoses are therefore managed by yearly or two yearly endoscopic and biopsy control. A diagnosis of HGD requires an immediate repeat endoscopy and biopsy to rule out an associated invasive adenocarcinoma (Miros M et al., 1991). Also, confirmation of the diagnosis by another experienced pathologist constitutes safe practice, due to the therapeutic implication of the diagnosis (Haggitt RC, 1994), and the significant interobserver variation reported in the diagnosis of dysplasia (Reid BJ et al., 1988a). There is, however, great controversy about the best form of management following a confirmed diagnosis of HGD and the exclusion of invasive adenocarcinoma. A group of investigators favors immediate esophagectomy (Miros M et al., 1991; Pera M et al. 1992; Rice TW et al., 1993; Altorki NK et al., 1991) due to the frequent association of HGD with adenocarcinoma (Miros M et al., 1991; Altorki NK et al., 1991; Schnell T et al., 1989), the frequent finding of invasive adenocarcinoma in esophagectomy specimens following a preoperative diagnosis of HGD (Rice TW et al., 1993), and the poor prognosis, even with curative surgical resection, once invasion has occurred in Barrett's adenocarcinoma (Witt TR et al., 1983). In contrast, another group favors a more conservative approach of frequent endoscopy and rigorous biopsy sampling of the entire BE at short intervals, while reserving surgery for confirmed progression into invasive adenocarcinoma (Rusch VW et al., 1994; Hameeteman W et al., 1989; Levine DS et al., 1993; Reid BJ et al., 1988b). This protocol is favored because of the relatively high operation-related mortality and morbidity rates following esophagectomy (Reid BJ et al., 1988b), the failure of some HGD to progress into invasive cancer after several years of follow-up (Rice TW et al., 1993), the demonstrated efficiency of endoscopic follow-up and systematic BE biopsy sampling to exclude presence of invasive adenocarcinoma (Reid BJ et al., 1988b; Levine DS et al., 1993; van Sandick JW et al. 1998), as well as the possibility, even though slight, of predicting biologic behavior of the preinvasive lesions through adjunctive methods like flow cytometry and mucin histochemistry (Haggitt RC et al. 1988; Reid BJ et al., 1988b; Rabinovitch PS et al., 1988; Lapertosa G et al., 1992). Experience with the two approaches to management is still limited at present to allow a reliable conclusion on the better of the two. A middle of the road approach has been provided by several new techniques like photodynamic therapy, Nd:YAG laser ablation and endoscopic mucosal resection (Inoue H et al., 1999). Endoscopic Nd:YAG laser ablation has been used successfully in the therapy of BE leading to subsequent squamous re-epithelialisation in an acid-free environment obtained by either anti-reflux surgery or omeprazole therapy (Salo JA et al., 1998). Similarly, photo induced ablation

of BE accomplished through photo activation of selectively localized photosensitizers led to partial re-epithelialisation of the BE by normal squamous epithelium in 68% of treated patients. In the same study, biopsy-proven complete remission of HGD was recorded in 100% of treated patients (Gossner L et al., 1998). Savary et al. employed photodynamic therapy in the treatment of early esophageal cancers and obtained impressive results (Savary JF et al., 1998). Following excellent results obtained with gastric cancers, endoscopic mucosal resection is now being used for the treatment of early esophageal cancers (Inoue H et al., 1999; Takeshita K et al., 1997). This technique has the advantage of allowing tissue submission for histopathologic confirmation of diagnosis and accurate determination of extent of disease. Survival rate of 95% was obtained at 5 years following endoscopic mucosal resection for early esophageal cancers (Inoue H, 1998).

The management of advanced Barrett's adenocarcinoma is not very much different from that of esophageal squamous cell carcinomas (Coia LR et al., 1999). Surgery and radiotherapy are the modalities for potentially curative therapy in localized diseased. Surgical resection can be through a left thoracic, a thoracoabdominal or a transhiatal esophagectomy with gastric, jejunal or colonic interposition or through an extrathoracic simultaneous abdominal-cervical laryngopharyngoesophagectomy (Streitz JM et al., 1990; Witt TR, 1983; Rusch VW et al., 1994). Radical surgery with subtotal esophagectomy and peritumoral resection to include the thoracic duct, lesser curvature of the stomach and extensive lymphadenectomy, produced better results (Lerut T et al., 1994). Therapy for patients with unresectable tumor or distant metastasis include, palliative surgery, external or internal radiation therapy (Witt TR et al., 1983; Green JRB et al., 1990), chemotherapy (Langer M et al., 1986), laser therapy (Carter R et al., 1990) or a combination of these (Morgan DG et al., 1990). Clinical prognostic factors identified in Barrett's adenocarcinoma include, stage on presentation (Sanfey H et al., 1985; Streitz JM et al., 1991), lymph node status (Lerut T et al., 1994) and presence of distant metastasis (Carter R et al., 1990). These are all a function of invasive disease and as such, the presence of invasive adenocarcinoma is a sine qua non for poor prognosis. For instance, in a study of 66 patients with Barrett's adenocarcinoma, 5-year survival rate was 100% for stage 0 and stage I tumors, but 0% for stage IV disease (Lerut T et al., 1994). Although there is currently a widespread use of endoscopic surveillance, majority of patients with Barrett's adenocarcinoma still present with advanced invasive disease (Fein R et al., 1985). This is possibly due to the propensity of Barrett's adenocarcinoma to metastasize while at the stage of intramucosal carcinoma, similar to the observation in gastric cancers. Therefore, surveillance in BE will be more effective when it is based on the search for a marker that not only

allows early detection of invasive cancer but that also allows a reliable prediction of the biologic behavior of the early dysplastic lesions in BE. Hence, the emphasis in BE research has shifted to the elucidation of the underlying molecular and genetic aberrations, which precede and determine the course of progression of the early preinvasive dysplastic lesions of BE (Bailey T et al., 1998).

In spite of all efforts, the prognosis for advanced stage cancers remains very poor with 5-year survival rates around 30% (Nishihira T et al., 1984). This is still so, even with resectable Barrett's cancers (Lerut T et al., 1994; Sanfey H et al., 1985; Streitz JM et al., 1991; Witt TR et al., 1983). The reason for this poor prognosis is multifactorial but most significant is the high rate of loco-regional tumor recurrence following potentially curative surgery or radiotherapy. This has been ascribed to the presence of micro metastases in the operating field or the presence of unrecognized distant metastasis at the time of primary therapy (Anderson and Lad, 1982; Watanabe H, 1997). In order to improve this poor picture, multimodality or combination therapy is being introduced. This can be by preoperative polychemotherapy with or without simultaneous radiotherapy followed by surgery. Alternatively, a non-surgical approach of maximal radiation dose and simultaneous polychemotherapy has also been tried. Several studies have shown that these modes of therapy lead to better results (Coia LR et al., 1999; Ilson and Kelsen, 1994; Langer M et al., 1986; Petrovich Z et al., 1989) with partial or complete remission of disease in up to 50% of patients (Ilson and Kelsen, 1994). This has, however, not resulted in significant improvement in survival rates because the reduced tumor associated mortality is compensated for by increased number of patients dying as a result of therapy associated complication (Anderson & Lad, 1982; Watanabe H, 1987). The realization that only patients with radio- or chemosensitive tumors will benefit from multimodality therapy informed the search for tumor associated factors, which can allow a reliable prediction of tumor sensitivity before initiating therapy. A few predictive factors have been identified in squamous cell carcinoma but not in adenocarcinoma of the esophagus. For instance, EGFR overexpression is predictive of poor response to radiotherapy (Hickey K et al., 1994); p53 gene abnormality is associated with reduced chemosensitivity and worse prognosis (Sarbia M et al., 1998; Ribeiro U et al., 1998) whereas cyclin D1 overexpression predicts poor survival with multi-modality therapy (Sarbia M et al., 1999a). Since the clinical detection of these predictive factors will be carried out on just a small portion of the tumor obtained before institution of therapy, the reliability of the findings greatly depend on how representative such a biopsy is of the entire tumor mass. The degree and pattern of intratumoral

heterogeneity within such a tumor will play a decisive role. Unfortunately, this has not been a focus of research in Barrett's adenocarcinoma.

1.6 Chromosomal and Genetic Aberrations in BE

1.6.1 Proliferative Abnormalities

Early attempts at characterizing the underlying genetic abnormalities in BE and its complicating preinvasive and invasive lesions involved indirect assessment of proliferation and cell kinetics. Evidence of expanded proliferative zone and increased proliferative activity was detected by autoradiography using tritiated thymidine (Herbst JJ et al., 1978; Pellish LJ et al., 1980). Immunohistochemical staining using monoclonal antibody against Ki-67, which recognized nuclear antigens in all stages of the cell cycle except G0, also demonstrated an expansion of the proliferative compartment, the severity of which correlated with the degree of dysplasia (Hong MK et al., 1995).

1.6.2 Chromosomal Anomalies

A more direct approach involved chromosomal and DNA content studies. By comparative genomic analysis, loss of the Y-chromosome has been found to be frequent and to correlate with progression in the metaplasia-dysplasia-carcinoma (MDC) sequence (Krishnadath K et al., 1995). Although this appeared to be an interesting finding when viewed against the background of predominant male affectation in BE and its neoplastic complications, the frequent occurrence of loss of Y-chromosome with advancing age makes the true value of this finding difficult to assess. Other frequent chromosomal abnormalities, which have been reported independently by different workers, include gain on 8q and 17q as well as losses on 18q and 4q (van Dekken H et al., 1999; Walch AK et al., 2000). The demonstration of similar chromosomal aberrations in contiguous areas of dysplasia and invasive adenocarcinoma has lent some credence to the proposed multistage process of Barrett's adenocarcinoma development (Gleeson CM et al., 1998). Also, the demonstration of similar cytogenetic abnormalities in adenocarcinoma and in regions of BE far removed from adenocarcinoma supports the clonal origin of neoplastic cells with subsequent expansion to involve extensive areas of the BE (Raskind WH et al., 1992). DNA content analysis using flow

cytometry further showed that raised G2/tetraploid fraction greater than 6% of the total cell population and the presence of aneuploidy was strongly correlated with the presence of dysplasia (Reid BJ et al., 1987) and is also predictive of progression into HGD or carcinoma (Reid BJ et al., 1992). Frequent occurrence of multiple aneuploidy was found in Barrett's adenocarcinoma (Rabinovitch PS et al., 1988) and in a large series of 80 patients, aneuploidy has been found to correlate significantly with presence of lymph node metastasis (Nakamura T et al., 1994).

1.6.3 Genetic Aberrations

Nowell among others, postulated that neoplasms develop as a clone from a single cell of origin with neoplastic progression resulting from selection of mutant subgroups (Nowell PC, 1976). An extension of this postulate came with the hypothesis of Knudson that oncogenesis requires some specific genetic mutations, which might be germinal or somatic, in some target cells (Knudson AG, 1993). These postulates have since been confirmed by many works, which demonstrated clonality of tumor cell population and the acquisition of specific karyotypic and genetic abnormalities occurring in a consistent pattern along with biological progression in neoplasms (Vogelstein B et al., 1989). It is this underlying principle that gave great impetus to the search for specific genetic anomalies that can characterize the histologically well-defined stages in the MDC sequence of BE. With the hope that these aberrations would allow a reliable prediction of biological behavior, virtually all classes of tumor associated genes are being investigated in BE and its associated complications.

1.6.3.1 Tumor Suppressor Genes (TSG)

TSG are important regulators of the normal cell cycle whose inactivation through deletion and or mutation is important in tumor development and prognosis (Vogelstein B et al. 1989; Michalides R, 1999). Numerous well defined and putative TSG have been described some of which have been mapped and cloned from specific chromosomal regions. Some of the most important TSGs frequently described in Barrett's adenocarcinomas are now considered in greater details.

P53 gene

p53 is a TSG located at the terminal part of the short arm of chromosome 17 (17p13), (McBride W et al., 1988). It plays a key role in the regulation of the cell cycle, by mediating the G1 cell cycle arrest after sublethal chromosomal damage and also by inducing apoptosis following severe irreparable DNA damage (Cordon-Cardon C, 1995). It has been implicated in the development of many different tumors where its mutation or the accumulation of its protein product has been demonstrated. In fact, p53 gene abnormality is the most frequent genetic abnormality detected in human tumors (Montessano R et al., 1996). In a review of more than 2500 tumor cell lines analyzed for p53 gene mutations, 45% of esophageal carcinomas, including adenocarcinomas, showed demonstrable genetic mutations and constituted the 3rd largest group of cancers with p53 gene abnormality (Ireland AP et al., 1997). In specific studies of Barrett's adenocarcinomas, p53 gene mutations and or p53 protein accumulation was demonstrated in a variable proportion of cases, with reported values ranging between 53% and 96% (Dunn J et al., 1999; Neshat K et al., 1994; Guido Coggi et al., 1997; Huang Y et al., 1992; Dolan K et al., 1998). Furthermore, a possible role for p53 gene in Barrett's adenocarcinoma tumorigenesis was indicated by the increasing frequency of 17p allelic deletion along with increasing dysplasia and adenocarcinoma in the MDC sequence of BE (Wu T et al., 1998; Blount PL et al., 1991). 17p allelic deletion in association with 18q deletion correlated with worse survival following multimodality therapy for Barrett's adenocarcinoma (Wu T et al., 1998), although a direct relationship between p53 gene mutation and response to surgical therapy was not demonstrable in several clinical studies (Ireland AP et al., 1997). Moreover, laboratory experiments designed to determine the effect of p53 gene mutation on response to possible adjuvant therapy like radiation and cytotoxic therapies, produced conflicting results (Lowe SW et al., 1994; Hawkins DS et al., 1996).

Retinoblastoma gene (Rb gene)

The Rb gene located on chromosomal band 13q14.2 was the first TSG to be discovered (Knudson AG, 1993). It functions as a cell cycle regulator by coding for a 105Kd nuclear phosphoprotein, whose underphosphorylated form binds transcription factors, like E2F, thereby preventing progression into the S-phase of the cell-cycle (Cordon-Cardo C, 1995). Because of its close interaction directly or indirectly with other cell-cycle regulators like CDK4, Cyclin D1, p53 and p21, its dysfunction results in uncontrollable cell proliferation, and predisposition to tumor development

(Fitzgerald RC & Triadafilopolous G, 1998). Rb gene abnormalities manifesting as LOH have been demonstrated in many human tumors including esophageal adenocarcinoma (Boynton RF et al., 1991; Dolan K et al., 1998). Although its mutation confers a worse prognosis in non-small cell carcinoma of the lung (Brambilla E et al., 1999) its specific role in BE progression and prognosis is still poorly elucidated.

Adenomatous polyposis coli gene (APC)

The APC gene is located on the long arm of chromosome 5 (5q21) (Knudson AG, 1993). Its primal role in the adenoma-carcinoma-sequence of colorectal adenocarcinoma made it the most plausible candidate responsible for the frequent LOH demonstrated on chromosome 5q in BE and its neoplastic complications as reported by different workers (Huang Y et al., 1992; Gleeson CM et al., 1998; Dolan K et al., 1998). The specific effect of abnormal APC gene product is not exactly known and was earlier thought to result directly from its binding to the catenins, which link the transmembrane E-cadherin adhesion molecules to the cytoskeletal actin filaments (Rubinfeld B et al., 1993; Su L et al., 1993). In support of this assumption, abnormal or reduced expression of alpha- and beta-catenins have been found in several human tumors including esophageal cancers (Takayama T et al., 1996; Shiozaki H et al., 1994) and was significantly correlated with the degree of differentiation in gastric carcinomas (Shiozaki H et al., 1991). Also, in vitro and in vivo studies showed that loss of E-cadherin adhesive function is associated with the acquisition of a dedifferentiated and invasive phenotype (Pignatelli MJ, 1993). Furthermore, a significant progressive reduction in E-cadherin was observed along with neoplastic progression in BE with aberrant expression of the catenins in Barrett's adenocarcinoma (Bailey T et al., 1998). Current knowledge has however, shown that the effects of APC gene mutation results in an abnormal product, which fails to bind ß-catenin. This leads to nuclear accumulation of this cytoskeletal protein which is then able to transactivate other nuclear phosphoproteins like c-myc thereby leading to genomic instability (Ilyas M & Tomlinson IPM, 1997; Arend JW 2000). LOH studies on premalignant and malignant BE has produced conflicting results on the temporal correlation of APC gene mutation and neoplastic transformation and progression in BE (Dolan K et al., 1999; Zhuang Z et al., 1996; Blount PL et al., 1993). Furthermore, mutational analysis in Barrett's adenocarcinoma revealed that APC gene mutation is a very rare event (Powell SM et al., 1994). These findings indicated the possibility of the presence of another gene on chromosome 5q, whose mutation could have resulted in the frequently observed 5q LOH in Barrett's adenocarcinoma.

Mutated in colon cancer gene (MCC)

This gene is localized to the same region 5q21 as the APC gene (Cripps KJ et al., 1998). Although the specific function of its protein product remains to be elucidated, the similarity between its predicted protein structure and that of the APC gene product, led to the assumption that the two products probably interact (Leggett BA et al., 1993). A high frequency of LOH mapped to this gene locus, has been reported in cardia and esophageal cancers, with values ranging between 63% and 75% of informative cases (Huang Y et al., 1992; Boynton RF et al., 1992).

1.6.3.2 Oncogenes and Growth Factors

Oncogenes are derived from proto-oncogenes, which are highly conserved, normal constituents of different species, where they play important role in cellular physiology (Spandidos DA & Anderson MLM, 1989). Proto-oncogenes can be divided into 3 classes: growth factor-coding proto-oncogenes, signal transducer-coding proto-oncogenes and proto-oncogenes that code for nuclear phosphoproteins (Jankowski J et al., 1992a). Conversion of a proto-oncogene to oncogene can occur following retroviral transduction, insertional mutagenesis, structural point mutations, chromosomal translocation or gene amplification (Spandidos DA & Anderson MLM, 1989). Abnormalities in proto-oncogenes have been demonstrated in different tumor types, including those of the gastrointestinal tract (GIT). Some of the important oncogenes that have been investigated in Barrett's adenocarcinoma include the following.

C-myc gene

This is the cellular homologue of the transforming gene of avian myelocytomatosis virus, whose translocation and abnormal expression are consistent findings in Burkitt's Lymphoma (Iizuka M et al., 1990). Amplification of this gene has also been reported in solid tumors of the GIT including the esophagus (Sarbia M et al., 1999; Brabletz T et al., 2000). Comparatively few studies have been carried out to elucidate the role of this oncogene in BE and its complicating lesions. Moreover, the few available reports were obtained using different techniques, which are not directly comparable. Thus, some workers found evidence for c-myc gene amplification in both preinvasive and invasive lesions of BE (Person DL et al., 1998; Abdelatif OMA et al., 1991; Lu S et al., 1998) while another failed to find evidence for this (Jankowsk i J et al., 1992a). Based on the

present level of knowledge therefore, it is difficult to make a categorical statement on the level and type of involvement of this gene in Barrett's adenocarcinoma tumorigenesis (Fitzgerald RC & Triadafilopolous G, 1998).

C-erbB-2 gene

The c-erbB-2 gene, also known as HER/neu gene, is located on the long arm of chromosome 17 (17q12-21.32) and encodes a 185kd transmembrane receptor with tyrosine kinase activity (Ross JS et al., 1999). It was originally identified as an oncogene in chemically induced rat neuroglioblastomas, in which a single point mutation in the transmembrane domain of the molecule confers oncogenic activation (Hengstler JG et al., 1999). A similar genetic mutation has not been found in human tumors (Hall PA et al., 1990; Lemoine NR et al., 1991) rather, gene amplification is the predominant mode of oncogenic conversion in humans. This is usually, but not always, associated with demonstrable membrane receptor overexpression (Lemoine NR et al., 1991; Slamon DJ et al., 1989). C-erbB-2 gene amplification or receptor overexpression has been found in a number of human adenocarcinoma from various body sites like pancreas, stomach, ovary, prostate and breast (Falck VG & Gullick WJ, 1989; Slamon DJ et al., 1989; Lemoine NR et al., 1991; Ross JF et al., 1999; Hall PA et al., 1999). A strong correlation exists between c-erbB-2 overexpression and poor prognosis in these cancers, especially in breast cancers, where it has led to the development of immunologic therapeutic agents directed at the c-erbB-2 receptor with significant impact on patient survival (Wisecaver JL, 1999). In vitro studies on esophageal adenocarcinoma cell lines showed c-erbB-2 gene amplification (Nishihira T et al., 1993). Also, native tumor samples showed gene amplification in 15.4% of cases (Al-Kaspooles M et al., 1993) and membrane receptor overexpression was found on immunohistochemistry in 19%, 26% and 73% of cases (Hardwick RH et al., 1995; Nakamura T et al., 1994; Jankowski et al., 1992a). In the study by Hardwick et al., the overexpression was confined to the adenocarcinoma tissue without involvement of the adjacent dysplastic lesion, a finding that suggested that c-erbB-2 abnormality might be a late event in Barrett's carcinogenesis. Although the number of studies in Barrett's adenocarcinoma is very low, compared with that in breast cancers, a similar association of c-erbB-2 receptor overexpression with poor prognosis has been reported in Barrett's adenocarcinoma by some workers (Nakamura T et al., 1994).

Epidermal growth factor receptor (EGFR) gene

The EGFR is a glycoprotein membrane receptor with tyrosine kinase activity. It is the binding site for 2 homologous growth factors, epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-alpha). EGF is a mitogenic polypeptide, which plays an important physiologic role in epithelial growth, maturation and healing (Wright NA et al., 1990). Likewise, TGF-alpha has been demonstrated in normal GIT mucosa, where it is localized to the differentiated superficial compartment (Thomas DM et al., 1992). Specific and reliable detection of EGFR in routinely processed paraffin embedded tissue using monoclonal antibodies is possible (Langbeheim H, 1990). By this means, significant increase in EGFR expression along with its ligand has been demonstrated in esophageal and gastric cancers (Mukaida H et al., 1991; Yoshida K et al., 1989), thus suggesting the possibility of an autocrine stimulation in carcinogenesis. Shiozaki et al. were able to demonstrate the ability of EGF to confer invasive property on esophageal cancer cell line in vitro (Shiozaki H et al., 1995). Using the same tumor cell line in an earlier study, Nishihira et al. also observed increased expression of mRNA for EGF and TGF-alpha along with amplification of the EGFR gene (Nishihira T et al., 1993). In addition to these experimental evidence for the involvement of EGFR and its ligands in carcinogenesis, clinical studies involving native tumor tissue also revealed EGFR gene amplification in esophageal and stomach cancers (Houldsworth J et al., 1990; Lemoine NR et al., 1991; Yoshida K et al., 1989; Lu S et al., 1988; Al-Kasspooles M et al., 1993). Furthermore, a progressive increase in EGFR protein overexpression was observed with increasing malignant behavior in gastric carcinomas (Koyama S et al., 1999), and overexpression of EGFR and its ligand, EGF, correlated with poor prognosis in esophageal cancers (Mukaida H et al., 1991).

1.6.3.3. Putative Tumor Suppressor Genes

Microsatellite markers are simple short repetitive DNA sequences distributed widely within the normal genome. These sequences can be expanded or deleted in tumor tissue, and then manifest as microsatellite instability (MSI) or as loss of heterozygosity (LOH) when tumor DNA is compared with matching normal DNA (Canzian F et al., 1996). MSI results from defects in the DNA mismatch repair gene, leading to reduced fidelity in the replication of repetitive DNA sequences and the appearance of new alleles in tumor tissue (Canzian F et al., 1996). In contrast, loss of genetic material through deletion, mitotic recombination, chromosomal non-disjunction or point

mutation manifests as LOH (Gupta PK et al., 1997; Tischfield JA, 1997). Whereas MSI is a frequent occurrence in colorectal carcinoma, particularly the hereditary non-polyposis colorectal cancers (HNPPC), where it has been found in up to 90% of cases (Bläker H et al., 1999; Dietmaier W et al., 1997), it is very rare in esophageal adenocarcinomas (Moskaluk CA & Rumpel CA, 1998). LOH, on the other hand, has taken a significant meaning following the two-hit hypothesis of Knudson (Knudson AG, 1993). Therefore, the recurrent loss of genetic material from specific chromosomal locations in a given tumor has been taken as evidence for the presence in the lost genetic material, of tumor suppressor gene involved in the genesis of the tumor (Gruis NA et al., 1993; Canzian F et al., 1996). Indeed, the use of microsatellite markers to detect LOH has led to the recognition of hitherto unknown TSGs or to the localization of existing ones (Emmert-Buck MR et al., 1997). The use of microsatellite markers has led to the detection of novel tumor associated genes in various tumors, for instance pancreatic adenocarcinoma.

Deleted in Pancreatic Carcinoma 4 (DPC-4) gene

Allelic loss on chromosome 18q has been demonstrated in cancers from several body sites including colon (Martinez-Lopez E et al., 1998), head and neck region (Papadimitrakopoulou VA et al., 1998), breast (Yokota T et al., 1997) and lung (Uchida K. et al., 1996). 18q loss has also been associated with tumor progression following in vitro studies on cell lines and native tumor tissue from SCC from the head and neck region (Frank CJ et al., 1997). Additionally, it correlated with poor prognosis in stage II colorectal cancers (Martinez-Lopez E et al., 1998). Several candidate TSGs map to this chromosomal region including DCC (Deleted in Colorectal Carcinoma), MADR-2 (Mothers against decapentaplegic related gene-2), and DPC-4. The last 2 genes are members of the SMAD family of genes and are involved in TGF-ß mediated control of cell cycle. Whereas DCC is a very large gene that does not lend itself easily to laboratory manipulation, DPC-4 has been localized to 18q21.1 with the aid of microsatellite markers through positional cloning. DPC-4 is altered in more than 50% of pancreatic adenocarcinomas (Hahn SA et al., 1996). Allelic loss involving this specific locus has also been demonstrated in up to 46% of premalignant and malignant Barrett's esophagus (Barrett MT et al., 1996b) but the clinicopathologic significance of this finding in Barrett's adenocarcinoma tumorigenesis is not yet clarified.

Allelic Loss on Chromosome 4

Loss of genetic material on the long arm of chromosome 4 detected either by microsatellite

allelotyping or by comparative genetic hybridization is a very frequent finding in esophageal adenocarcinoma (Gleeson CM et al., 1998; Hammoud ZT et al., 1996; Rumpel CA et al., 1999). The same aberration has been described in breast cancers and in hepatocellular carcinoma (Tirkkonen M et al., 1997). Frequent occurrence of genetic loss at a particular locus is a pointer to the existence of a TSG mapped to this locus and the frequent occurrence of such an abnormality in a particular type of cancer indicates that the suspected TSG plays an essential role in the development of the tumor. Despite the high frequency of demonstrable abnormality on chromosome 4q in several different cancers, however, no TSG has been mapped to this region except for the finding of an earlier in vitro experimental study, which ascribed a senescence function to this region (Ning Y et al., 1991). Notwithstanding, a reproducible 4q allelic loss in more than 50% of esophageal adenocarcinoma strongly suggests the presence of a TSG that may play an important role in Barrett's adenocarcinoma tumorigenesis.

1.7 Genetic heterogeneity in Barrett's Adenocarcinoma

Genetic heterogeneity in tumor cell population is a recognized feature of many human solid cancers. The prevalent notion that tumors consist of a homogenous population of abnormal proliferating cells followed the postulate of Nowell that tumors develop through a clonal proliferation of abnormal cells (Nowell PC 1976). Progression is then believed to result from subsequent acquisition of additional abnormalities in a subpopulation of tumor cells, which acquire a selective growth advantage, thus becoming the predominant cell population. In this linear progression model, tumor characteristics like invasion and metastasis are believed to be late events dependent on the accumulation of numerous genetic anomalies. The Knudson's two-hit hypothesis (Knudson AG 1993) and the dysplasia-carcinoma sequence of colorectal carcinogenesis further strengthened this theory by providing a genetic model for tumor initiation and progression. In as much as the clonal origin of tumor cells has been corroborated by various works employing X-chromosome inactivation pattern to determine cell lineage, recent researches especially on human solid cancers have shown that tumors are made up of a heterogeneous population of cells. Rather than a clone with growth advantage being selected, tumor progression is now thought, at least in a subset of human tumors, to occur by a process of clonal diversity. This implies that tumors consist of different clones of cells, which share some genetic abnormalities because of their origin from a common progenitor cell, but also with peculiar genetic anomalies restricted to certain clones. Thus, a set of

genetic aberrations may lead to selective growth advantage in some subclones in the primary tumor, while another subclone with different genetic aberrations may possess metastatic potential. In this regard, matching studies between primary tumors and metastatic foci revealed that metastatic clones may represent an early clonally divergent population and not necessarily the result of a simple linear progression in tumors (Fujii H et al., 1996; Bläker H et al., 1999; Kuukasjärvi T et al., 1997). Using a variety of methods like flow cytometry, chromosomal and genomic analysis as well as molecular genetic analysis, many workers have demonstrated the occurrence of significant intratumoral heterogeneity in different human solid cancers like breast (Lichy JH et al., 2000; Aubele M et al., 1999), urinary bladder (Takahashi T et al., 1998; Diaz-Cano SJ et al., 2000) and prostate (Macintosh CA et al., 1998; Suzuki H et al., 1998). A clonal origin with subsequent expansion has been suggested for BE following the observation of a progressively expanding clone of cells over a segment of BE (Raskind WH et al., 1992). However, neoplastic progression in BE is associated with genomic instability (Rabinovitch PS et al., 1988) and BE, especially with dysplasia is heterogeneous consisting of a mosaic of cells (Kumble S et al., 1996; Galipeau PC et al., 1999). Nonetheless, van Dekken and co-workers were able to show that BA is monoclonal in origin, even when multifocal (van Dekken H et al. 1999). Very few studies have been devoted to the elucidation of heterogeneity in esophageal cancers, and even then, these are mostly on squamous cancers using flow cytometry to study variation in DNA content from different parts of a tumor (Sasaki K et al., 1991; Haraguchi Y et al., 1995). Flow cytometry suffers from the disadvantages that small variations in DNA cannot be detected and no specific information regarding the involved chromosomal region or genetic loci is obtained (Voorter C et al., 1995). It is therefore important to investigate the presence of heterogeneity in Barrett's adenocarcinoma using a method that is sensitive and specific enough to detect small variations in genetic constituent but also simple enough to facilitate quick transfer of the findings into clinical usage. It is the aim of this study to investigate genetic heterogeneity in Barrett's adenocarcinoma using polymorphic markers to detect allelic loss at TSG loci and amplification at some proto-oncogene loci, which have been reported in esophageal and other GIT solid neoplasms.

1.8 Research Objective

Barrett's adenocarcinoma is associated with a very poor prognosis mainly due to its propensity to metastasize early, and the high frequency of loco-regional tumor recurrence after seemingly curative surgical resection. There is therefore a great need both for early diagnosis and for multimodality therapy, which has been shown to improve prognosis in advanced stage cancers but only in a subset of patient population. Molecular markers offer the best means for early recognition of cancers and for identifying potential patients who could benefit from multimodality therapy. Hence, the great search for reliable tumor markers in Barrett's adenocarcinoma. The effective use of these molecular markers in patient management will require the collection of a preoperative biopsy, which is usually obtained through an incisional biopsy from the superficial surface of the Barrett's lesion. This, however, may not be representative of the tumor cell population on the deep invading front. Furthermore, there is a great impetus for unraveling the molecular pathogenesis of Barrett's adenocarcinoma, similar to attempts being made with other human cancers. This often involves microdissection of representative tumor tissue, usually from different parts of the tumor. Intratumoral heterogeneity has been demonstrated in various human cancers from the prostate, liver, breast, but absent in a subset of bladder cancers. This phenomenon has been shown to be capable of not only impeding the detection of significant anomalies, but may also lead to false conclusions. Thus, the above mentioned therapeutic and investigative processes concerning Barrett's adenocarcinoma are liable to be adversely affected by the presence of heterogeneity in Barrett's adenocarcinoma. It is therefore the aim of this study to investigate the occurrence of heterogeneity in Barrett's adenocarcinoma, the degree of this heterogeneity and the presence of any topographic pattern to this heterogeneity. The findings of this study will be of value in the management of Barrett's adenocarcinoma patients and also in many on-going research activities devoted to elucidating the molecular characteristics of Barrett's adenocarcinoma.

2. Materials and Methods

2.1 Appliances and Reagents

2.1.1 Appliances

Table 1

Name	Manufacturer
Autoclaving Machine	Melag
Densitometer	Bio-Rad
Dissecting Microscope	Olympus
Electronic Weighing Balance	Bio-Rad
Electrophoresis Tank (Agarose Gel)	Biometra
Electrophoresis Tank (Polyacrylamide Gel)	Bio-Rad
Gel Drier	Bio-Rad
Heat Incubator	Eppendorf
Micropipettes	Eppendorf
Microwave	Miele
PCR Machine	Biometra
Table Centrifuge	Denver Scientific
Table Centrifuge (Vortex)	Heidolph
Thermomixer	Eppendorf
UV-Light Source	AGS GmbH
Video Copy Processor	Mitsubishi

2.1.2 Reagents

2.1.2.1 Chemicals

Table 2

Name	Firm
Acetic Acid (100%)	Merck
Agarose Gel Powder	GIBCO BRL
Ammoniumpersulfate	Merck
Base Pair Marker	GIBCO BRL
Boric Acid	Merck
Bromophenol Blue	Sigma
Deionised Distilled Water	Braun
dNTPs	Qiagen
EDTA	Merck
Ethanol (100%)	Riedel-de Haen
Ethidium Bromide	Sigma
Formaldehyde (Formalin 37%)	Merck
Formamide	Merck
Glycerine	Sigma
Hydrochloric Acid	Merck
PCR-Buffer	Qiagen
Polyacrylamide Gel Stock Solution	Bio-Rad
Silver Nitrate	Merck
Sodium Carbonate	Merck
Sodium Hydroxide	Merck
TEMED N,N,N,N-tetramethylethylenediamene	Bio-Rad
Tris	Merck
Urea	National Diagnostics
Xylencyanol	Merck

2.1.2.2 Buffer and Solutions

Table 3

Buffer/Solution	Constituents and Concentration
Gel Loading Buffer (Agarose Gel)	0.25% Bromophenol blue 0.25% Xylencyanol 30.0% Glycerin
Gel Loading Buffer (Polyacrylamide Gel)	95% Formamid 0.05% Bromophenol blue 0.05% Xylencyanol 20mM EDTA, PH 8.0
10X PCR-Buffer	Tris/HCL, PH 8.7 (20%) Kcl $(NH_4)_2SO_4$ 15mM $MgCl_2$
Silver Stain: Color Development Lotion	0.5% Formaldehyde 1.5% Sodium Hydroxide
Silver Stain: Fixing Lotion	10% Ethanol 1% Acetic Acid
Silver Stain: Neutralising Lotion	0.75% Sodium Carbonate
Silver Stain: Staining Lotion	0.2% Silver Nitrate
1X TBE-Buffer	0.09M Tris/HCl, PH 8.0 0.09M Boric Acid 0.002M EDTA
1X TE-Buffer	10mM Tris/HCl, PH 7.5 0.1mM EDTA

2.1.3 Enzymes

Table 4

Enzyme	Manufacturer
Proteinase K	Merck
Restriction Enzyme (RsaI)	Boehringer Mannheim
Restriction Enzyme (XbaI)	Boehringer Mannheim
Taq-Polymerase	Qiagen
Taq-Polymerase (Hotstar)	Qiagen

2.1.4 PCR-Primers

Table 5

Gene	Chromo-some	Direction 5° - 3°	Sequence	Firm
APC	5q	upstream downstream	GGA CTA CAG GCC ATT GCA GAA GC TAC ATC TCC AAA AGT CAA	MWG-Biotech
APRT	16q	upstream downstream	TGG GAA AGC TGT TTA CTG CG CAG GGA ACA CAT TCC TTT GC	MWG-Biotech
C-erbB-2	17q	upstream downstream	CCT CTG ACG TCC ATC ATC TC ATC TTC TGC TGC CGT CGC TT	MWG-Biotech
C-MYC	8q	upstream downstream	CCT CAA CGT TAG CTT CAC CAA C CTG CTG GTA GAA GTT CTC CTC	MWG-Biotech
D4S1652	4q	upstream downstream	AAT CCC TGG GTA CAT TAT ATT TG CAG ACA TTC TTT ATT CTT TAC CTC C	MWG-Biotech
D18S474	18q	upstream downstream	TGG GGT GTT TAC CAG CAT C TGG CTT TCA ATG TCA GAA GG	MWG-Biotech
EGFR	7p	upstream downstream	AGC CAT GCC CGC ATT AGC TC AAA GGA ATG CAA CTT CCC AA	MWG-Biotech
IFN	12p	upstream downstream	GCA GAG CCA AAT TGT CTC CT GGT CTC CAC ACT CTT TTG GA	MWG-Biotech
MCC	5q	upstream downstream	TAC GAA TCC AAT GCC ACA CTG AAG TAG CTC CAA ACA	MWG-Biotech
RB	13q	upstream downstream	CTG CAG TCC CAC CTC AGC CTC CTT AGT AGA GGA TCC GCA GCT CTA GAC TAA TCC CAG CAC	MWG-Biotech

2.2 Patient Collective and Test Material

2.2.1 Patient Collective

Materials obtained from 10 patients managed at the surgical unit of the Heinrich-Heine-University Hospital, Düsseldorf, were employed in this study. The patients, all male with ages at the time of surgery ranging between 41 years and 73 years and a mean and median of 62 years and 55 years respectively, were not subjected to any form of preoperative radio- or chemotherapy.

2.2.2 Test Materials

Materials consisted of archival collection of paraffin embedded tissue sections from esophagectomy specimens of the 10 patients with histologically confirmed Barrett's adenocarcinoma of the esophagus. All the resected tumors were cut serially into 5 mm thick longitudinal sections and embedded completely in paraffin. The resected materials were handled between 1991 and 1999 at the Institute of Pathology, Heinrich Heine University, Düsseldorf. Prior gross morphologic description on routine handling showed that 5 tumors were ulcerated in appearance, 3 were polypoid and the remaining 2 showed a mixed ulcerated/polypoid appearance. The widest tumor diameters ranged between 35mm and 65 mm with a mean of 52.8 mm and a median of 55 mm. (Table 6).

2.3 Histologic Examination and Selection of Material

Using 5µm-thick, hematoxylin and eosin (H&E) stained sections prepared from the completely embedded longitudinal tissue sections, an experienced pathologist with special interest in esophageal cancers (PD Dr. med. M. Sarbia), reconfirmed the initial diagnosis of Barrett's adenocarcinoma and also ascertained the tumor grade and stage. Tumor grading, using WHO grading system (WHO 1990), showed three grade 2 tumors, six grade 3 tumors, and one grade 4 tumor. Staging was carried out using the UICC classification system (Wittekind and Sobin 1997) whereby 2 of the cases were in stage II, 7 in stage III and the tenth was a stage IVA tumor. There was failure to achieve curative resection in 3 of the cases. A detailed presentation of patient and tumor characteristics is shown in Table 6. The final selection of cases and corresponding paraffin embedded tissue sections from the archival collection was positively influenced by the presence of lymph node metastasis and a high preponderance of tumor cells relative to contaminating stromal or

Table 6 **Patient and Tumor Characteristics**

Case NR.	Sex	Age in Years	Gross Appearance	Tumor Size	Grade	Tumor Status	Nodal Status	Metas-tasis	Stage
1	M	58	ulcer	55 mm	G3	pT2	pN1	pMX	II
2	M	70	polyp	55 mm	G3	pT3	pN1	pMX	III
3	M	61	polyp/ulcer	60 mm	G3	pT3	pN1	pM1a	IVA
4	M	50	polyp	48 mm	G2	pT2	pN1	pMX	IIB
5	M	73	polyp	60 mm	G2	pT3	pN1	pMX	III
6	M	73	ulcer	50 mm	G3	pT3	pN1	pMX	III
7	M	54	ulcer	35 mm	G2	pT3	pN1	pMX	III
8	M	65	ulcer	55 mm	G3	pT3	pN1	pMX	III
9	M	41	ulcer	45 mm	G4	pT3	pN1	pMX	III
10	M	55	polyp/ulcer	65 mm	G3	pT3	pN1	pMX	III

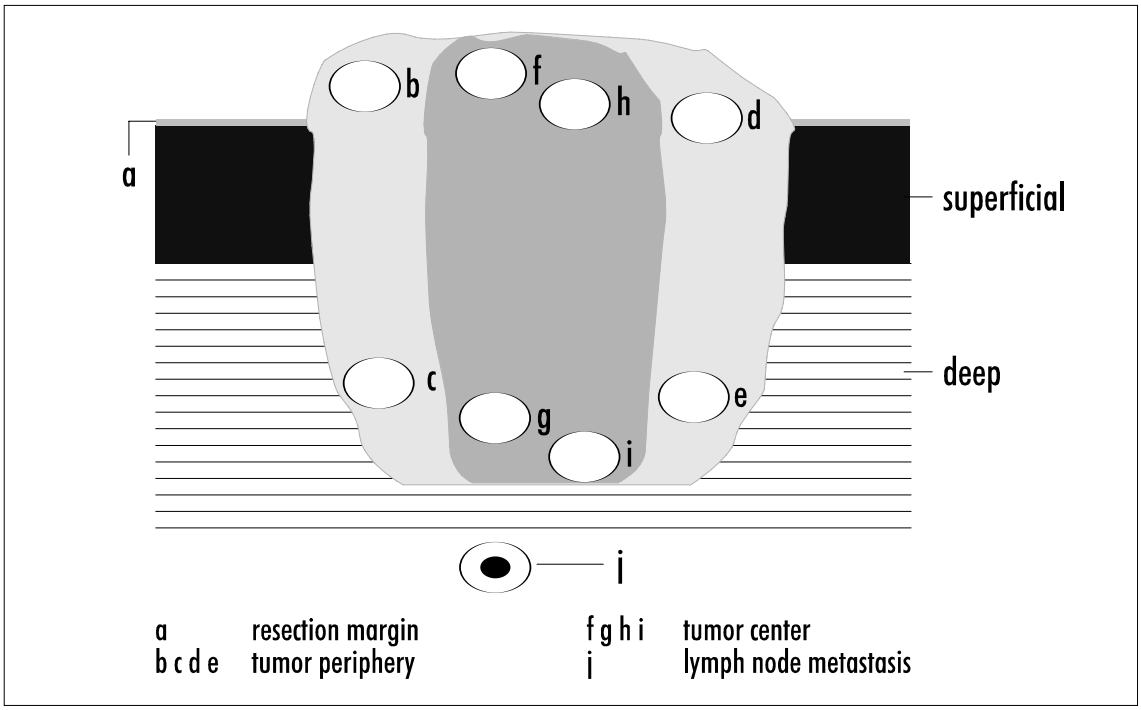


Fig. 5 Graphic presentation of the microdissected areas from each of the 10 cases.

inflammatory cells. This enabled the retrieval of relatively homogenous but highly enriched specimen (at least 70% tumor cell content) from different tissue regions consisting of 10 distinct areas (a-j) (fig. 5).

2.4 DNA Preparation

Following the selection of suitable histologic sections, DNA preparation was carried out following previously published protocols (Bianchi AB et al., 1991; Zhuang Z et al., 1995; Moskaluk CA & Kern SE 1997) with slight modifications as follows. From each selected paraffin block, two serial 10µm-thick tissue sections, freshly produced by microtome sectioning, were mounted on glass slides, deparaffinised twice with xylene, rinsed thrice in decreasing grades of ethanol, rinsed in water, stained briefly with hematoxylin and finally rinsed and maintained in deionised water until ready for scrapping. An additional 5µm-thick section immediately adjacent to the previous sections was prepared to serve as reference slide. The reference slide was H&E stained and coverslipped as per standard histological techniques. The areas of interest were rechecked

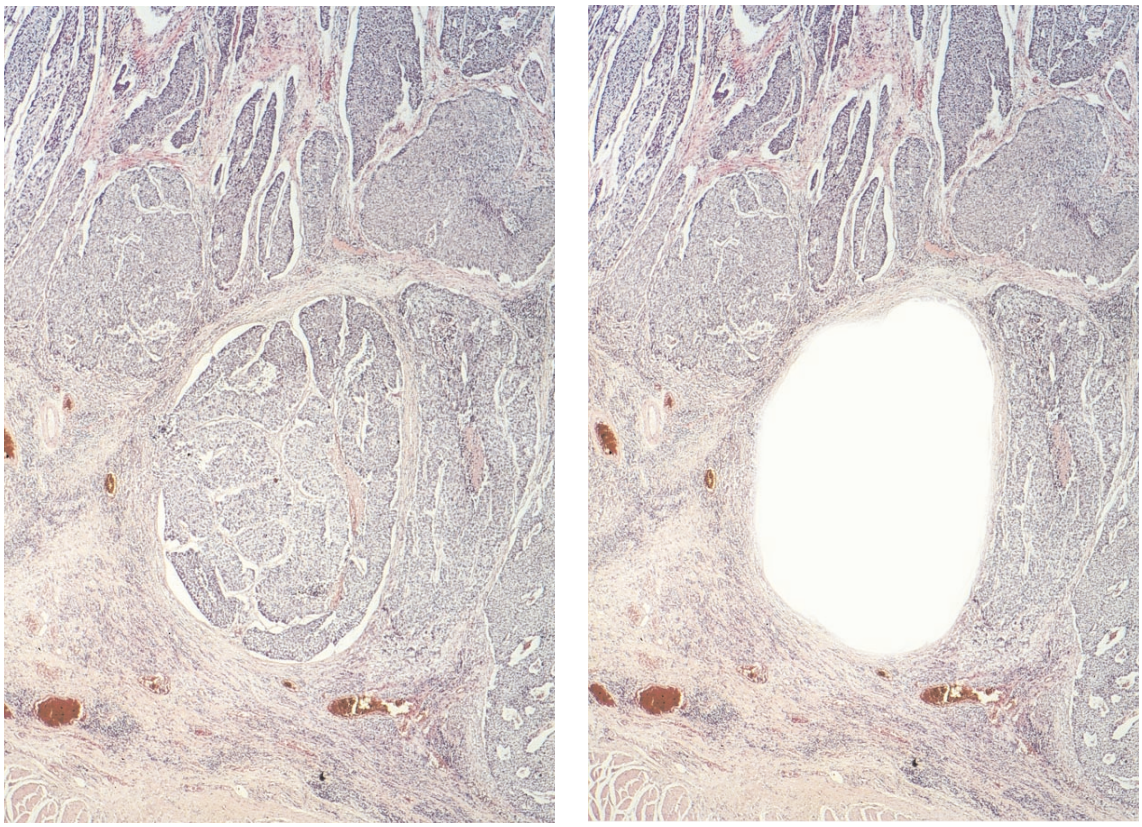


Fig. 6 Nests of invasive tumor cells before (left) and after (right) microdissection (poorly differentiated adenocarcinoma).

under the microscope and demarcated by encircling with an indelible marker (Faber Castell) on the reference slide. Using the reference slides as guide, fractional tissue microdissection from the corresponding areas on the 10µm-thick sections was carried out with a sterile ophthalmic scalpel under microscopic control (Olympus Japan). (see fig. 6 showing microdissection of tumor tissue). The scalpel was resterilized by flaming after each scraping to avoid tissue to tissue contamination. The procured tissue was immediately placed in TE-buffer (50-200 ml), depending on the amount of tissue. Tissue digestion using proteinase K (1 mg/ml) at a ratio of 1:10 relative to TE-buffer, was carried out overnight at 55^0C in a thermomixer (Eppendorf, Germany). Inactivation of excess enzyme after tissue digestion was effected by heating the preparation to 94^0C for 8min. The preparations were centrifuged briefly to bring down condensate. Between 2 and 5µl of the supernatant was employed as DNA templates in the following polymerase chain reactions (PCR) without further purification.

2.5 Molecular Genetic Examinations

2.5.1 Polymerase Chain Reactions (PCR)

The PCRs for amplifying the products of APC, D4S1625, D18S474, MCC and RB gene loci were carried out in a total volume of 25µl or 50µl after several initial trials to determine the optimal PCR conditions and the appropriate concentrations for the various constituents of the PCR mixture. Each PCR included a negative control, in which the DNA-template was replaced with sterile distilled water. Extracted DNA either from a cell line (OSC1, or A431), or from paraffin embedded tumor tissue known to be positive for LOH at the tested locus served as positive controls for the PCRs. The respective PCR condition, primer sequences and the PCR mixture concentration for each of the tested gene loci is presented in table 7.

2.5.2 Differential Polymerase Chain Reactions (dPCR)

dPCR was carried out to detect amplification at the c-myc, c-erbB-2 and EGFR gene loci. This involved simultaneous amplification of the tested gene with a reference gene, which was either an 82-bp fragment interferon (IFN), located on chromosome 12 and/or adenosine phosphoribosyl transferase (APRT) located on chromosome 16. The optimal primer concentrations and the dPCR

Table 7 Details of the PCR-Mixture Constituents and PCR Parameters at Different Gene Loci.

Gene Loci	PCR Constituents and Concentrations.								PCR Condition	Remarks
	DNA template	DNA Taq-Poly-merase	Buffer	dNTP	Target Gene A&B primers	Ref. Gene (IFN/aprt) A&B primers	Dist H_20	Total Vol.		
APC	2-10µl	0.5µl	5µl	1µl	0.5µl	NA	32.5µl	50µl	Initial Denaturation. 94^{0}C-4 min; (Denaturation: 94^{0}C-1 min, Annealing: 55^{0}C-45s; Polymerization: 72^{0}C-2 min)x35 cycles; Final Elongation: 72^{0}C-6 min	
D4S1652	2-10µl	0.25µl	5µl	1µl	0.5µl	NA	32.75 µl	50µl	Initial Denaturation. 94^{0}C-4 min; (Denaturation: 94^{0}C-1 min, Annealing: 58^{0}C-1 min; Polymerization: 7^{2}0C-1 min)x35 cycles; Final Elongation: 72^{0}C-7 min	
D18S474	2-5µl	0.13µl	2.5µl	0.5µl	0.25µl	NA	16.37 µl	25µl	Initial Denaturation. 94^{0}C-4 min; (Denaturation: 94^{0}C-1 min, Annealing: 58^{0}C-1 min; Polymerization: 7^{2}0C-1 min)x35 cycles; Final Elongation: 72^{0}C-7 min	
MCC	2-10µl	0.5µl	5µl	1µl	0.5µl	NA	32.5µl	50µl	Initial Denaturation. 94^{0}C-9 min; (Denaturation: 94^{0}C-1 min, Annealing: 53^{0}C-45s; Polymerization: 72^{0}C-2 min)x35 cycles; Final Elongation: 72^{0}C-6 min	
RB	2-10µl	0.5µl	5µl	1µl	0.5µl	NA	27.5µl	50µl	Initial Denaturation. 94^{0}C-9 min; (Denaturation: 94^{0}C-1 min, Annealing: 62^{0}C-1 min; Polymerization: 72^{0}C-2 min)x30 cycles; Final Elongation: 72^{0}C-8min	Hotstar Taq Polymerase
C-MYC	2-10µl	0.6µl	5µl	1µl	3µl/1.5µl	3µl/1.5µl	14.4µl	50µl	Initial Denaturation. 94^{0}C-5 min; (Denaturation: 94^{0}C-1 min, Annealing: 56^{0}C-1 min; Polymerization: 72^{0}C-1 min)x31 cycles; Final Elongation: 72^{0}C-4 min	IFN = 1.5µl APRT = 3µl
C-erbB-2	2-10µl	0.6µl	5µl	1µl	2µl	1µl	17.4µl	50µl	Initial Denaturation. 94^{0}C-5 min; (Denaturation: 94^{0}C-1 min, Annealing: 56^{0}C-1 min; Polymerization: 72^{0}C-1 min)x30 cycles; Final Elongation: 72^{0}C-4 min	
EGFR	2-10µl	0.6µl	5µl	1µl	2µl	1µl	17.4µl	50µl	Initial Denaturation. 94^{0}C-5 min; (Denaturation: 94^{0}C-1 min, Annealing: 56^{0}C-1 min; Polymerization: 72^{0}C-1 min)x31 cycles; Final Elongation: 72^{0}C-4 min	Hotstar Taq Polymerase

NA: not applicable

parameters were first established for each primer pair, so that selective amplification of one PCR fragment at the expense of the other was eliminated. Also, the number of cycles was maintained very low to ensure that the dPCR did not proceed beyond the exponential phase of amplification, so as not to obscure any potential amplification. In addition, a set of positive controls were amplified along with the tested probes. These consisted of extracted DNA from cell lines, COLO 320DM for c-myc (Sarbia M et al. 1999b), A431 for EGFR (Yamamoto T et al. 1986), and SKBR-3 for c-erb-B2 (An H et al. 1995) as well as paraffin embedded tumor tissue with known amplification for the tested gene loci. Placental tissue served as reference tissue with normal copy number of the tested gene. The details of the dPCR condition and the contents and concentrations of PCR mixtures are presented in table 7.

2.5.3 Agarose Gel Electrophoresis (AGE)

In order to confirm successful PCR amplification at the investigated loci, AGE (3%) was carried out after every PCR. To this end, 2.4g agarose powder was dissolved in 80ml of 1X TBE-buffer by boiling in a microwave oven. This was followed by the addition of 7µl ethidium bromide with gentle stirring before pouring into a gel container. Electrophoresis wells were created by inserting a gel-comb with the appropriate number of teeth into the gel before it has cooled down to about 60°C for polymerization. Following polymerization, the gel was transferred into the electrophoresis tank containing 1X TBE-buffer. A portion of the PCR-product, about 10µl, was mixed with 2µl agar-gel-loading-buffer and then loaded into the electrophoresis wells with the aid of micropipettes (Eppendorf, Germany). Each electrophoresis also included an extra well, filled with 10µl of 100 bp DNA as size standard. The electrophoretic separation of the products was carried out at 80 mV for 80 min. Thereafter, the gel was exposed to UV-light (=302nm) and photographed, using a video copy processor (Mitsubishi, Japan).

2.5.4 Polyacrylamide Gel Electrophoresis (PGE)

The detection of loss of heterozygosity was carried out by subjecting the PCR products, which were initially detected on AGE, to a further process of PGE which shows a better separation and higher sensitivity than AGE. The gel plates were cleaned using 70% alcohol and then mounted on the gel holding rack (Biometra) without any further preparation. The contents of the gel included

10 ml stock solution of 40% polyacrylamide (acrylamide:bisacrylamide; 29:11), 65µl of TEMED, 6 ml of 5X TBE, 650µl ammonium persulfate, and 14.4g urea. After ensuring total dissolution of the urea by vigorous shaking, the gel was poured into the mounted gel plates and allowed to polymerize with the gel comb inserted. After 1 hour of polymerization, the gel was transferred into the electrophoresis chamber (Miniprotean, Biometra) containing 1X TBE-buffer. The gel comb was removed and an initial phase of electrophoresis with empty wells was carried out for 15 minutes. Thereafter, the wells were carefully rinsed with TBE buffer and filled with 2-5µl PCR-product mixed with 1µl polyacrylamide gel loading buffer. A preceding denaturation step at 95°C for 5 min was first carried out for the PCR-products of the D4S1625 and D18S474 gene loci, whereas the products of the APC and RB gene loci underwent a 3-hour restriction enzyme digestion with RsaI and Xba1 restriction enzymes respectively. There was no special handling for the MCC gene product before PGE. Electrophoresis proceeded at 150 mV for 2 hours, after which the PGE chamber was dismantled and the gel placed in a fixing lotion for 20 minutes for proper fixation prior to staining. Following fixation, the gel was gently rinsed in distilled water for about 5 minutes before being placed in the silver staining lotion for 20 minutes. Subsequent handling of the gel entailed washing off excess silver lotion for about 5 minutes in distilled water, color development in the alkaline development lotion for about 3-10 minutes, gentle rinse in distilled water before stopping the color development by placing in a weakly acidic neutralizing lotion. Finally, a last round rinsing in distilled water was carried out before drying the gel under vacuum at 80^{O}C for 30 minutes.

2.6 Analyses

2.6.1 LOH Analyses

Assessment of LOH was carried out by visual inspection of the silver stained and dried polyacrylamide gel. Cases were first assessed as informative i.e. heterozygous or non-informative i.e. homozygous. Only heterozygous cases were suitable for LOH analysis. LOH was scored as present when there is a complete loss of an allelic copy or a significant reduction in allele/allele ratio in any of the tumor tissues (b-j) compared with the ratio in the corresponding normal tissue (a). Each run of PGE included a pair of normal and carcinoma tissue with demonstrable LOH serving as control. All cases with LOH and all cases with equivocal results were subjected to at least one more round of PCR followed by AGE and PGE. Only reproducible cases of LOH were eventually scored as positive for LOH.

In order to determine the clonality of the tumors,all analyses showing LOH were specifically assessed to determine the specific copy of the allelic pair that was lost in tumor tissue from different regions of a tumor. Thus, the loss of the same copy of an allelic pair across different regions of a tumor is indicative of a common origin for the tumor cells and thus implies monoclonal tumor origin (Cheng L et al., 1996). (fig. 7)

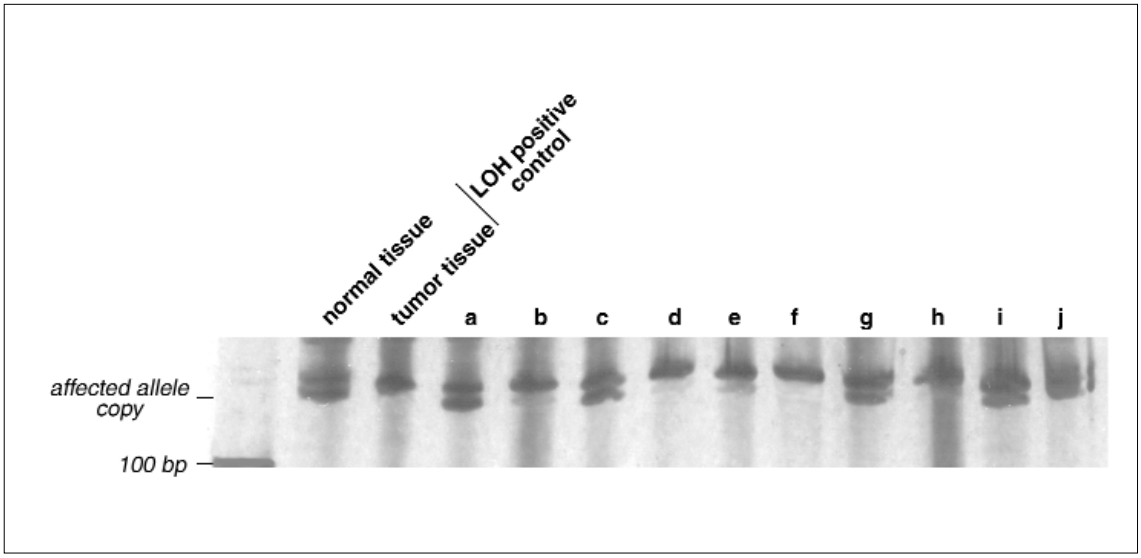


Fig.7 Case of LOH at the D4S1652 gene locus, note the loss of allele copy in lanes b, d, e, f and h compared with lane a. The same allele copy was affected at all the sites.

2.6.2 Gene Amplification Analysis

Assessment of gene amplification was carried out on photographed agarose gels by laser scanning and densitometric quantification of the signal intensity using the ScanPack 2 computer software (Biometra). The validity of the dPCR was first established by ascertaining that the ratio of signal intensity of target gene to that of reference gene (IFN) is at least 3-fold higher in positive controls compared with negative control (placenta). Thereafter, a similar comparison was carried out between the ratios obtained from each of the 9 tumor regions and that obtained from the accompanying normal tissue. Tumor areas showing at least a 3-fold increase over and above the value obtained for corresponding normal tissue were regarded as amplified. Amplification was verified by repeating the dPCR and densitometry and finally confirmed with a third dPCR using a different reference gene (APRT). Only cases which could be reproducibly verified and confirmed in this manner were accepted as amplified. (fig. 8).

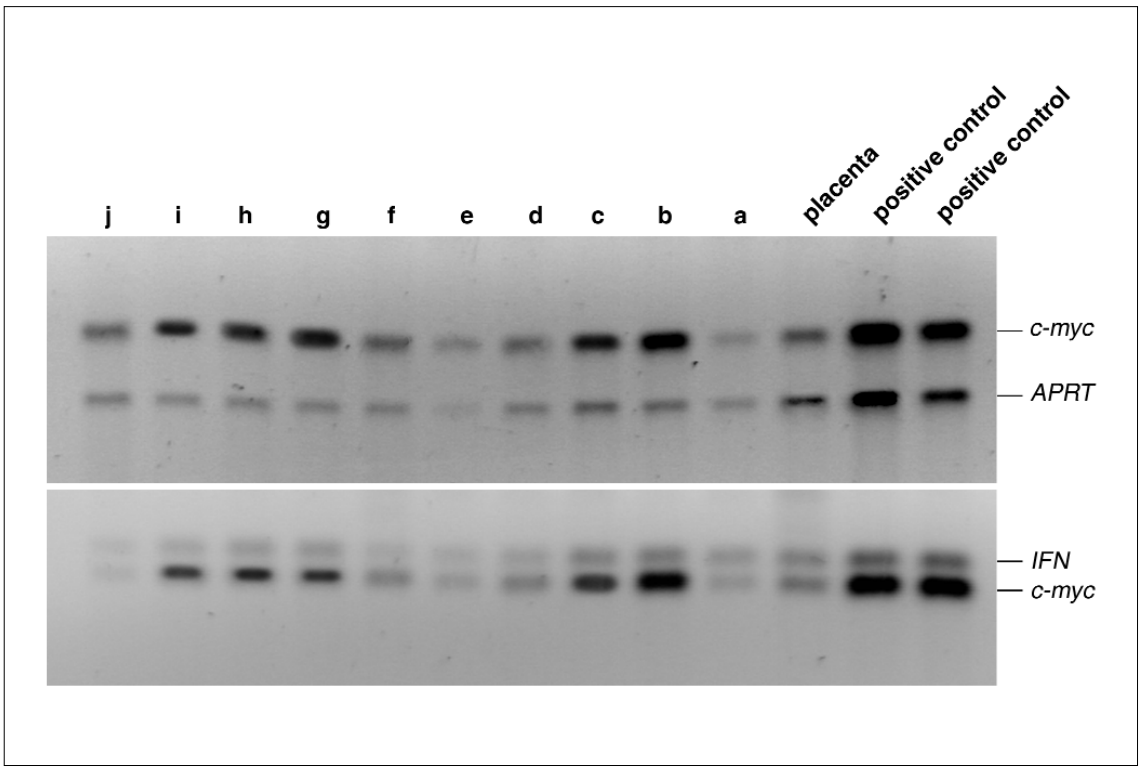


Fig.8 C-myc gene amplification (lanes b, c, g, h and i) demonstrated by dPCR using APRT (upper picture) and IFN (lower picture) as reference genes.

3. Results

Ten cases of Barrett's adenocarcinoma were investigated for genetic abnormalities at 8 different gene loci making a total of 80 analyses. Out of these, 67 analyses were informative or suitable for detection of genetic abnormalities and for assessing the pattern of distribution of these abnormalities within the tumors and thus suitable for the detection of intratumoral heterogeneity. In contrast, 12 analyses which were homozygous at different gene loci were unsuitable for LOH analysis. Repeated dPCR did not produce any appreciable product with the c-myc gene primers in 1 case, despite successful amplification of DNA from accompanying positive and placenta controls. The detailed results of genetic anomalies obtained at the different loci in all the tumors are shown in Table 8 and in a graphicform in Table 9).

Table 8

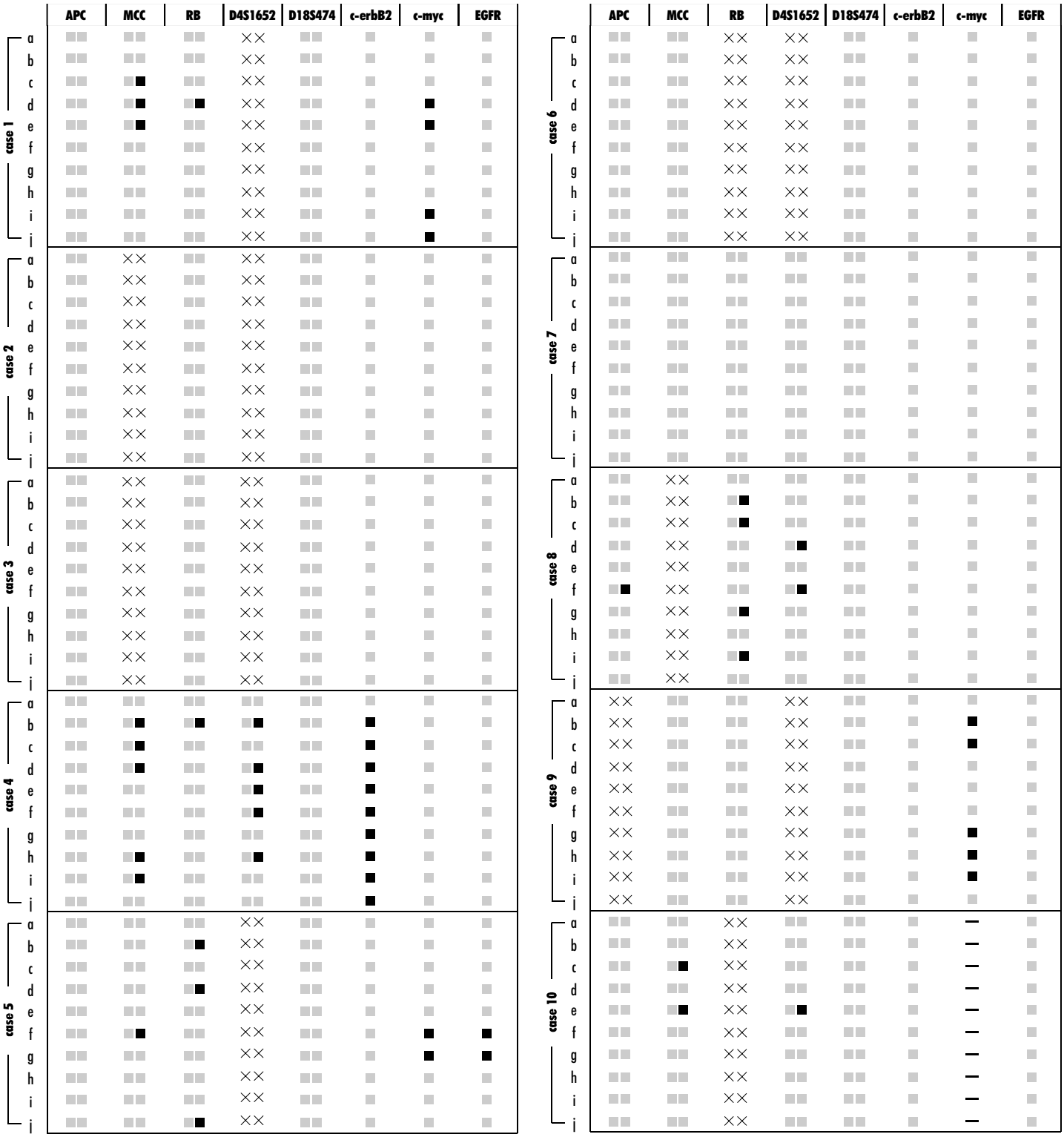
Informativity, LOH, amplification and heterogeneity rates at different gene loci.

Gene Loci	Informative Cases	Amplification/ LOH rate	Tumor Center		Tumor Periphery		Lymph Node	Heterogeneity rate
			Superficial[1]	Deep[1]	Superficial[1]	Deep[1]		
APC	9/10 (90%)	1/9 11%	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/1 (0%)	100%
D4S1652	4/10 (40%)	3/4 (75%)	3/6 (50%)	0/6 (0%)	3/6 (50%)	2/6 (33%)	0/3 (0%)	100%
D18S474	10/10 (100%)	0/10 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	-
MCC	7/10 (70%)	4/7 (57%)	2/8 (25%)	1/8 (13%)	3/8 (38%)	5/8 (63%)	0/4 (0%)	100%
RB	8/10 (80%)	4/8 (50%)	2/8 (25%)	0/8 (0%)	4/8 (50%)	3/8 (38%)	1/4 (25%)	100%
C-ERBB2	10/10 (100%)	1/10 (10%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/1 (100%)	0%
C-MYC	9/10 (90%)	3/9 (33%)	2/6 (33%)	4/6 (67%)	2/6 (33%)	2/6 (33%)	1/3 (33%)	100%
EGFR	10/10 (100%)	1/10 (10%)	1/2 (50%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/1 (0%)	100%
Total	67/80 (84%)	17/67 (25%)	13/34 (38%)	8/34 (24%)	14/34 (41%)	14/34 (41%)	3/17 (18%)	

[1] : 2 tissue samples were analyzed per case at each of these sites.

Table 9

Genetic abnormalities at 8 different gene loci in the 10 investigated cases of Barrett's Adenocarcinoma.



a: normal tissue, b - i: tumor tissue from the primary, j: local lymph node metastasis (see figure 5).

3.1 LOH Results

3.1.1 APC

Nine of 10 cases (90%) were informative for LOH analysis. One out of 9 informative cases revealed LOH (11%). In this single case, LOH was observed at only 1 of the 9 tumor regions investigated (superficial region, tumor center); an indication of the heterogeneous distribution of this genetic abnormality within the tumor.

3.1.2 D4S1652

Four of the 10 cases studied were informative giving an informativity rate of 40%. Three out of these 4 cases showed LOH (75%), and in these 3 cases, LOH was heterogeneously distributed within the tumor tissues, thus giving a heterogeneity rate of 100%.

3.1.3 D18S474

All the cases were informative for analysis but none of them showed LOH, thereby giving an informativity and LOH rates of 100% and 0% respectively. It was not possible to assess intratu moral heterogeneity at this locus.

3.1.4 MCC

Seven of 10 cases were heterozygous, 70% informativity rate. LOH was detected in 4 of the 7 informative cases, giving a 57% LOH rate. A heterogeneous distribution of LOH was found in all the tumors giving a heterogeneity rate of 100%.

3.1.5 RB

The RB gene was heterozygous in 8 out of the 10 cases studied, with 80% informativity rate. Four of these 8 cases showed LOH with heterogeneous distribution, thereby giving 50% and 100% LOH and heterogeneity rates respectively. (fig. 9).

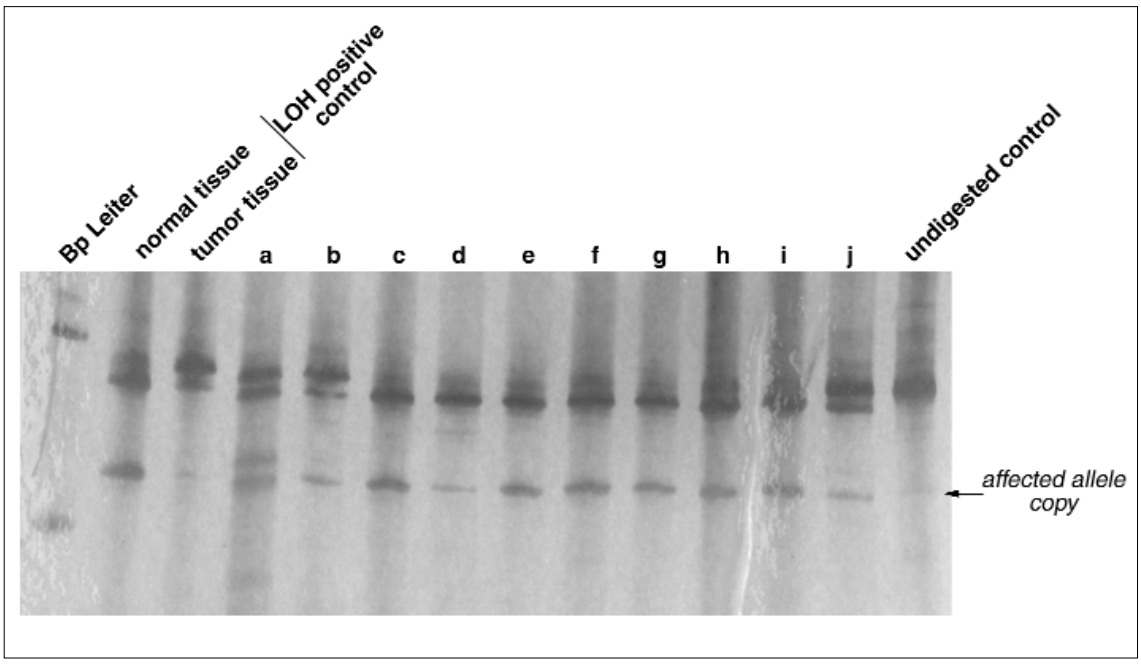


Fig.9 Case of LOH at the Rb gene locus showing loss of the same allele copy at all the sites positive for LOH (b, d, j).

3.2. Gene Amplification

3.2.1 C-erbB-2

Only 1 of the 10 cases investigated in this study showed c-erbB-2 gene amplification, giving an amplification rate of 10%. Amplification was detectable at all the different sites of the tumor tissue including the lymph node metastasis. This implies a homogenous distribution of this genetic abnormality. The tumor therefore showed no intratumoral heterogeneity at this locus. (fig. 10).

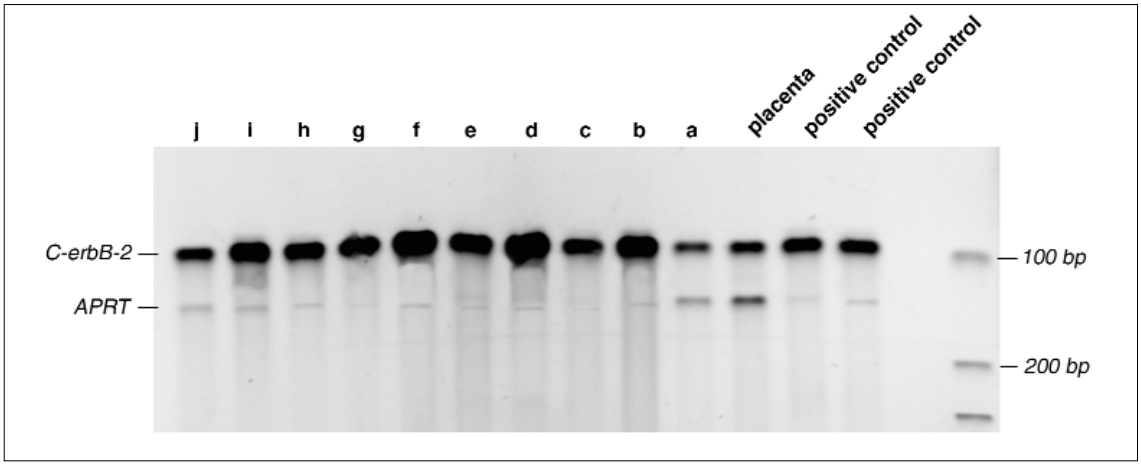


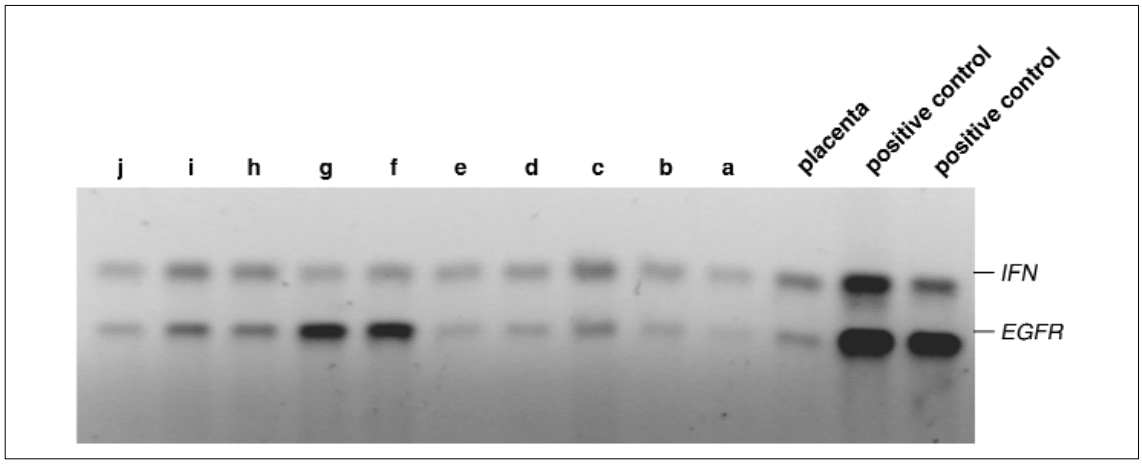
Fig.10 dPCR showing homogeneous c-erbB-2 gene amplification at all the tested tumor sites (b-j). Note tumor free resection margin (a) and placenta without gene amplification.

3.2.2 C-myc

Reliable dPCR was only possible in 9 of the 10 cases studied whereas sufficient PCR product could not be obtained with the last case probably due to DNA degradation. Three of the 9 successfully amplified cases showed c-myc gene amplification, all with a heterogeneous pattern. Thus giving an amplification rate of 33% and heterogeneity rate of 100%.

3.2.3 EGFR

Out of 10 cases subjected to dPCR, only 1 case revealed evident EGFR gene amplification and only at 2 contiguous sites from the center of the tumor. Amplification rate at this locus, therefore, came to 10% and heterogeneity rate was 100%. (fig. 11).



dPCR showing heterogeneous EGFR gene amplification (lanes f & g). The same case showed c-myc gene co-amplification restricted to the same intratumoral sites (see picture below).

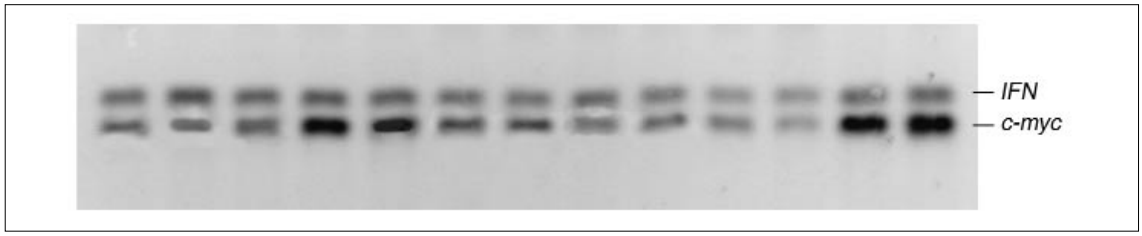


Fig.11 c-myc gene amplification restricted to lanes f & g similar to picture above showing EGFR amplification restricted to the same sites.

3.3 Intratumoral Heterogeneity

Seventeen of the 67 informative analyses revealed genetic anomalies. Of these 17 cases,16 cases (94%), showed genetic heterogeneity whereby the detected abnormalities were limited to just some of the tested tumor sites (see figs. 7, 8, 9 and 11). The highest rate of positivity within

any particular tumor with heterogeneity was not more than 5 tumor areas. A single case showed homogeneous presence of c-erb-B2 amplification at all the 9 tumor sites. (see fig. 10)

3.4 Tumor Clonality

Twelve cases of LOH were detected in this study. An analysis of the allelic loss pattern in all the cases showed that the same allele copy was lost at all the positive sites within each positive tumor. This indicated that all cases with LOH in this study were monoclonal in origin. (figs. 7 & 9).

4. Discussion

Intratumoral genetic heterogeneity has been studied in other tumor types apart from Barrett's adenocarcinoma by several workers using different techniques like comparative genomic hybridization, LOH analysis, single strand conformation polymorphism (SSCP) analysis and gene sequencing. These different methods have their relative advantages and disadvantages. The choice of LOH and gene amplification analysis for this study was informed by the need to use a method sensitive enough to detect minimal genetic aberrations in a small proportion of tumor cells, specific enough to allow proper localization of the observed anomaly to any of the known genetic loci implicated in Barrett's adenocarcinoma carcinogenesis and simple enough to allow quick investigation of a large number of samples. LOH analysis and dPCR to detect gene amplification best fulfilled these criteria. These methods required DNA preparation and amplification, which are known to be best carried out with fresh tissue. Due to the retrospective nature of this study, however, archival paraffin-embedded materials ranging between 1 and 9 years old were used. This is not likely to have adversely affected the results of this study significantly since reliable and reproducible PCR had been carried out using paraffin-embedded tissue (Bianchi AB et al., 1991) even where this was 10 years old (Gruis NA et al., 1993).

Furthermore, the technique of fractional microdissection used in this study, implied that relatively little amount of tissue was collected for DNA extraction, a factor that could lead to insufficient DNA extraction. However, that this was not the case is borne out by the fact that it was possible to detect PCR products reproducibly in all the cases and from all the sites using 8 different markers. The only exception was a single case with failed dPCR amplification using c-myc gene primers. This could not have resulted from insufficient DNA since the same case was successfully amplified using 7 other primers for the other gene loci tested with no incidence of allelic dropout in the accompanying normal tissue, the hallmark of insufficient DNA content (Zhuang Z et al., 1995). It has actually been shown that reliable PCR amplification is possible with as little as 36 pg genomic DNA, equivalent to DNA content of only 3 cells (Berthon P et al., 1995).

Although a comparable study of this nature has not been carried out on Barrett's adenocarcinoma, the LOH rates detected at 3 of the 5 tested loci ranged between 50% and 75%. These rates were considerably higher than the background LOH rates of 23% reported from a comprehensive allelotyping of esophageal adenocarcinoma (Barrett MT et al., 1996a). Hence, it is safe to conclude that the technique employed in this study was sensitive enough to have detected aberrations at all the tested tissue sites, were these to harbor any aberration.

The following detailed analysis and comparison of findings in this study with previous reports by other workers should further buttress this point.

The APC/MCC genes co-localized on the long arm of chromosome 5 constitute one of the most investigated gene loci in gastrointestinal tumor studies including the esophagus. Various authors have investigated LOH involving both loci in esophageal carcinomas. These include Boynton et al. who reported a combined rate of LOH at both loci in 75% of 16 informative adenocarcinomas of the esophagus (Boynton RF et al., 1992). Huang et al. in a combined study of esophageal squamous and adenocarcinoma reported 66% and 63% LOH rate at the APC and MCC loci respectively (Huang Y et al., 1992), whereas Dolan and co-workers detected 45% LOH rate in esophageal adenocarcinoma using multiple microsatellite markers mapped to 5q (Dolan K et al., 1998). Contrarily, Powell et al. using in-vitro synthesized protein assay, SSCP and gene sequencing, found out that APC gene mutation is very rare in esophageal adenocarcinomas, occurring in only 1 of 18 cases studied (Powell SM et al., 1994). Since inactivation of TSGs commonly involves deletion of an allele with accompanying mutation of the other allele, LOH detects an early inactivating event in TSGs. Thus, the APC-LOH in 1 of 9 informative cases (11%) in this study is comparable to the finding of Powell et al. although the methods employed were different. However, it is very low when compared with findings from the other cited authors who employed LOH analysis as used in this study. Discrepant APC-LOH rates from different authors have generated a lot of controversy in the past leading to suggestion that perhaps an entirely different gene also maps to 5q that could be responsible for the observed LOH (Powell SM et al., 1994). In this respect, a novel TSG on 5q described in lung carcinomas supports this position (Wieland I & Bohm M, 1994). It is however, important to recognize the fact that the rates provided by Boynton and Dolan groups contained both MCC and APC-LOH lumped together. Thus, the possibility remains to be excluded that the high LOH rates in these reports were not mainly contributed by MCC-LOH. Furthermore, notwithstanding the strong case that has been made by several studies for a role for APC gene in the malignant progression of BE (Zhuang Z et al., 1996; Dolan K et al., 1999), it is still not entirely impossible that APC gene aberration is not obligatory in a subset of these tumors. Several studies on gastric and colorectal lesions, where APC gene inactivation is very frequent, revealed some subsets of patients and tumors without demonstrable APC gene abnormality (Fang D et al. 1999; Peltomaki P et al. 1992; Sud R et al. 1996). But for the fractional microdissection

technique employed in this study, the only case with APC-LOH would not have been detected, because the abnormality was detected in only 1 of the 9 different sites investigated in this particular tumor. Hence, it is a likely possibility that a subset of Barrett's adenocarcinoma may not require APC gene inactivation for neoplastic transformation and progression.
The 57% MCC-LOH rate in this study is comparable with the 63% reported by Huang et al. who also observed a significant association between MCC-LOH and LOH at other loci like p53, RB and DCC but not with APC (Huang Y et al., 1992). In keeping with their observation, the current study also showed that MCC-LOH was frequently associated with LOH at other gene loci with the exception of APC gene locus. This association is particularly remarkable because almost all the cases showing MCC-LOH showed multiple genetic aberrations in at least 2 other gene loci but not APC. The few cases with multiple aberrations without associated MCC-LOH were homozygous for MCC, so that the status of this gene could not be determined using the technique of LOH analysis. Although the small number of cases in this study precluded statistical analysis, it is difficult to completely agree with the proposition of Huang et al. that MCC-LOH is a result and not the cause of the frequently observed association of other genetic aberrations with MCC-LOH. Further research on the basic molecular function of the MCC protein should throw more light on this aspect of the findings.

The RB gene is involved in cell cycle control through its interaction with such G1 phase control molecules like CDK4 protein and Cyclin D1. LOH involving this locus was reported in between 36% and 48% of esophageal carcinomas (Boynton RF et al., 1991; Huang Y et al., 1992). In 2 separate series involving 17 and 23 esophageal adenocarcinomas, Dolan et al. reported LOH rates 21% and 31% respectively (Dolan K et al. 1998; Dolan K et al. 1999). The LOH rate of 50% in this study is generally slightly higher than those from these previous reports. The higher rate obtained in this study probably reflected the higher likelihood of detecting genetic abnormalities by sampling multiple different tumor areas. Although no significant role has been ascribed to this gene in Barrett's adenocarcinoma tumorigenesis, the demonstration of 13q allelic loss in premalignant BE by Gleeson et al. (Gleeson CM et al., 1998) suggests that it may play a role in the early stages of BE neoplastic transformation and progression.

DPC-4-LOH was found in none of 10 informative cases, using a microsatellite marker D18S474 that is specific for this gene. This marker is one of the most frequently employed markers at this locus (Martinez-Lopez E et al., 1998; Papadimitrakopoulou VA et al., 1998; Yokota T et al., 1997; Schutte M et al., 1996) and was even employed in the positional cloning of the gene (Hahn SA et

al., 1996). The failure to detect LOH at this locus in the present study may be due to one or other of the following. (1). It may indicate that DPC-4 gene is not the target of 18q loss, therefore its inactivation may not be important for Barrett's adenocarcinoma tumorigenesis. This is a likely possibility because mutational analysis in esophageal and other GIT adenocarcinomas (Lei J et al., 1996; Barrett MT et al., 1996b) as well as in head and neck SCC where frequent 18q loss has been observed, also revealed infrequent DPC-4 mutation (Kim SK et al., 1996). (2). It could be the effect of random selection, whereby we selected a subset of tumors in which DPC-4 aberration has not occurred. DPC-4 inactivation occurs most commonly by homologous deletion (Hahn et al., 1996) and has been demonstrated to be a late event in ovarian and pancreatic carcinogenesis (Wilentz RE et al., 2000). Although biallelic deletion is technically difficult to detect from native tumor tissue as employed in this study, due to possible contaminating non-neoplastic cell population (Hoque ATMS et al., 1997), LOH analysis is suitable for detecting the initial step of single allelic deletion preceding the biallelic inactivation. It is therefore not totally impossible that this finding resulted from random selection bias in favor of tumors without DPC-4 aberration, a difficulty which is almost impossible to overcome. Noteworthy however, is the finding of LOH even in premalignant Barrett lesion in an earlier mentioned work by Barrett and co-workers (Barrett MT et al., 1996b). They employed 3 microsatellite markers including the one used in this study. Unfortunately, the authors failed to provide details about the LOH rate for each marker. Perhaps the selection of another microsatellite marker could have revealed LOH at this gene loci because it was revealed that the distance of a marker from the suspected gene can significantly influence the rate of LOH observed (Cairns P et al., 1994).

4q allelic loss was investigated using the microsatellite marker, D4S1652, mapped to the long arm of chromosome 4. LOH was detected in 3 of 4 informative cases giving a rate of 75%, the highest LOH rate detected at any locus in this study. This is in accord with the findings by Gleeson et al. who found evidence of 4q allelic imbalance in 8 out of 11 (73%) informative Barrett's adenocarcinomas using the same marker, D4S1652, employed in this study (Gleeson CM et al., 1998). Most works on Barrett's adenocarcinomas are focused on established genes, which have been implicated in cancers from other sites, thereby making the detection of novel genes which might play unique role in Barrett's carcinogenesis less likely. Thus, only a few other chromosomal studies have demonstrated frequent 4q allelic loss in esophageal adenocarcinoma (Hammoud ZT et al., 1996; Rumpel CA et al. 1999). The present study is of great value because it has further highlighted the significance of the findings from the few previous works that inves-

tigated 4q allelic loss in Barrett's esophagus. It has also given additional support for the possibility of a TSG on 4q which may play an important role in esophageal adenocarcinomas. An earlier in vitro experimental study ascribed a senescence function to this chromosomal region (Ning Y et al., 1991) but no known gene has been mapped to this site. Therefore, a detailed deletional mapping study on 4q will be a worthwhile step that could unravel any putative TSG mapped to this region.

Evidence for gene amplification was found at all the 3 oncogene loci investigated using dPCR. EGFR gene amplification was detected in 1 out of 10 cases giving a rate of 10%. Also, this abnormality was distributed in a heterogeneous manner, being limited to only 2 adjacent superficial and deep sites from the center of the tumor. Different groups using blotting technique or differential PCR have reported rates of EGFR gene amplification in esophageal cancers ranging between 3.6% and 30.8% (Al-Kasspooles M et al., 1993; Houldsworth J et al., 1990; Hollstein et al., 1988; Lu S et al., 1988). The rate of 10% obtained in the current work therefore falls well within previously reported rates and is quite close to the 8% and 14% reported by Hollstein and Lu groups respectively. The low rate of 3.6% by Houldsworth et al. might have resulted from the dilutional effect of the gastric tumors included in the study since infrequent EGFR gene amplification has been demonstrated in a small series of gastric cancers (Lemoine NR et al., 1991). Co-amplification of oncogenes was previously observed in gastric and esophageal carcinomas (Yoshida et al., 1991; Al-Kasspooles M et al., 1993) and has been suggested to result from synergistic selection during tumor progression. The only tumor with EGFR gene amplification in this study also showed simultaneous amplification of the c-myc proto-oncogene. Remarkably enough, the amplification observed with both genes was restricted to exactly the same sites within the tumor. A similar co-amplification involving both genes was detected in a human gastric cancer by Nomura et al. (Nomura N et al., 1986). This is not likely to be a random event and thus, strongly suggests a synergistic role for these genes in the progression of this tumor. In this regard, it is instructive to note the correlation between EGFR overexpression and poor prognosis in several human cancers like breast, cervix and esophagus (Slamon DJ et al., 1989; Mukaida H et al., 1991). C-erbB-2 gene amplification was also found in 1 out of 10 cases studied giving a rate of 10%. Previously reported rates using dPCR technique were 15.4% (Al-Kasspooles M et al., 1993) and 10.7% (Houldsworth J et al., 1990) both of which are comparable to the rate obtained in this study. Considerably higher rates of 19%, 26% and 73% were obtained by other authors who used immunohistochemistry to detect membrane overexpression of the c-erbB-2 protein (Nakamura T et al.,

1994; Hardwick RH et al., 1995; Jankowski et al., 1992a). C-erbB-2 gene amplification correlates well with membrane protein overexpression and is the predominant mode of up-regulating the gene in humans (Wisecarver JL, 1999). Nevertheless, membrane protein overexpression in the presence of a normal gene copy number has been demonstrated in human tumor tissues (Slamon DJ et al., 1989) where it probably resulted from transcriptional up-regulation. The possibility of this mechanism resulting in increased expression was reported in some gastric cancers where over-expression resulted from the action of a protein, which binds the TATA sequence in the promoter region of the c-erbB-2 gene (Kameda T et al. 1990). This mechanism of transcriptional upregulation, although subordinate to gene amplification in humans, might have contributed to the higher rates obtained on immunohistochemistry. The frequent occurrence of a genetic abnormality in a particular tumor provides the basis for implicating such a gene in the pathogenesis of the tumor, thus c-erbB-2 gene abnormality has been implicated in Barrett's adenocarcinoma. However, the discrepant results obtained by immunohistochemical detection of c-erbB-2 protein in premalignant and malignant BE has led to controversy regarding the time course of action of c-erbB-2 in BE neoplastic transformation and progression (Hardwick RH et al., 1995) i.e. whether it is an early or late genetic event in Barrett's carcinogenesis. The homogenous pattern of c-erbB-2 gene amplification detected in this study strongly indicates that the abnormality occurred early in the malignant transformation of BE. It most likely occurred before the onset of clonal diversity and heterogeneity, which was revealed in the same tumor by the heterogeneous distribution of abnormalities at the D4S1652, MCC and RB gene loci. In addition, the persistence of the amplification throughout the tumor suggests that it may also be important in tumor progression. For instance, c-erbB-2 protein overexpression has been correlated with poor prognosis in many human cancers like breast cancer (An H et al., 1995), ovarian cancer (Ross JS et al., 1999) and also esophageal cancer (Nakamura T et al., 1994). The prognostic significance of c-erbB-2 protein overexpression in breast cancers led to the successful introduction of monoclonal antibodies directed against an epitope contained within its gene product in the treatment of breast cancer patients (Wisecarver JL, 1999). Further elucidation of the function of this gene and its abnormalities in Barrett's adenocarcinoma may provide similar opportunity for therapy in esophageal cancers.

Three out of 9 suitably amplified cases showed a heterogeneous c-myc gene amplification giving a rate of 33.3%. This is comparable to the 26.1% obtained by dPCR in squamous cell carcinoma of the esophagus (Sarbia M et al., 1999b). It is, however, higher than the 14% (3 of 22 esopha-

geal cancers) reported by Lu et al. using southern blotting technique (Lu S et al., 1988). The lower rate in the latter study may be due to the higher level of contamination with normal cells associated with the southern blot technique. Although the significance of c-myc gene amplification in Barrett's adenocarcinoma tumorigenesis is still poorly elucidated, the demonstration of this aberration in dysplastic BE but not in BE devoid of dysplasia (Abdelatif OMA et al., 1991) as well as in adjacent non-tumorous epithelium surrounding esophageal cancers (Lu S et al. 1988) supports an early role for this genetic abnormality in esophageal cancers. Furthermore, an experimental study showed that suppression of endogenous c-myc expression led to reduced proliferation and increased differentiation (Yokoyama K & Imamoto F, 1987). It is therefore remarkable, that the only G4 tumor included in this study was one of the three showing c-myc gene amplification. The specific role of this gene in Barrett carcinogenesis needs more detailed clinicopathologic study, but being the most frequent oncogene aberration detected in this work, its significance has been further highlighted.

A total of 67 analyses at 8 different loci were informative in this study, of which 17 revealed genetic abnormalities either LOH or gene amplification. The intratumoral distribution of abnormalities in 16 of these 17 (94%) instances was heterogeneous, which shows that Barrett's adenocarcinoma is comprised of very heterogeneous population of cells. This heterogeneous distribution of abnormalities could, however, have resulted from 1 or the other of 3 possibilities like (1) technical factor leading to failure to detect abnormalities at many of the investigated sites; (2) fusion of multiple independent multiclonal tumors or (3) clonal diversity within a monoclonal tumor.

(1) This finding is not likely to have resulted from technical factors for the following reasons. Six of the 10 cases investigated revealed a heterogeneously distributed genetic abnormality in at least 1 of the 8 gene loci tested. Abnormality was detected in 7 of the 8 gene loci investigated and usually in different cases or in different combination of cases where the abnormality was present in more than a single case. Where multiple aberrations were present in a case, the intratumoral distribution of abnormality varied with different gene loci. Thus showing that the abnormalities were not restricted to a set of cases or markers, and also not restricted to any particular sites within a particular tumor.

(2) Multifocality and field cancerization is a recognized phenomenon in cancers of the upper aerodigestive airway, whereby the exposure of a wide area of epithelium to similar carcinogenic agents could lead to the development of multiple independent synchronous or metachronous cancers (Franklin WA et al., 1997). The fact that BE can cover an extensive surface area makes the

possibility of field cancerization a likely possibility, with a greater likelihood of multifocal tumor and recurrence. Thus several workers have attempted to determine the origin and clonality of neoplastic cells in Barrett's adenocarcinoma. The traditional method for determining clonality of tumor cell population through X-chromosome inactivation pattern is unsuitable due to the low frequency of Barrett's adenocarcinomas in females. Nevertheless, other comparable albeit, less specific methods have been employed. For instance, van Dekken et al. using comparative genomic hybridization evaluated a multifocal adenocarcinoma of the esophagus and was able to show that the multiple foci of tumor shared majority of the observed genomic aberrations and thus, could only have resulted from a monoclonal tumor cell population (van Dekken H et al., 1999). Similarly, Robaszkiewicz analyzed multiple samples from 6 Barrett' adenocarcinomas with flow cytometry and concluded that Barrett's adenocarcinoma arose from a single clone of cells (Robaszkiewicz M et al., 1992). Allelic loss pattern using microsatellite markers is another method for determining clonality, whereby the deletion of the same copy of an allele in separately microdissected areas of a tumor is an indication of monoclonality of the tumor cells (Cheng L et al., 1998). This method has been shown to be comparable to X-chromosome inactivation pattern in terms of specificity and was employed to demonstrate clonality of 2 Barrett's adenocarcinoma cases (Zhuang Z et al., 1996). Analysis of the allelic loss pattern in all the cases with LOH in this study revealed that the same copy of the tested allele was deleted at all the positive sites from each case. This finding has therefore excluded multiclonality as the cause of the observed heterogeneous distribution of genetic abnormalities in this study. Moreover, the opportunity of analyzing 6 different cases with LOH at 5 different genetic loci makes this study about the largest study of clonality in Barrett's adenocarcinomas so far and has further confirmed the findings of other workers that Barrett's adenocarcinoma is mainly of monoclonal origin.

(3) The exclusion of the other 2 possibilities leaves clonal diversity within a monoclonal tumor as the possible cause of the observed heterogeneity in this study. Only very few studies have been devoted to the study of intratumoral heterogeneity in esophageal cancers and especially so in Barrett's adenocarcinoma (Haraguchi Y et al., 1995; Robaszkiewicz M et al., 1992; Sasaki K et al., 1991). Using flow cytometry to study DNA content of different regions of tumors, Robaszkiewicz et al. demonstrated intratumoral heterogeneity in esophageal adenocarcinoma (Robaszkiewicz M et al., 1992). The present study has been able to confirm their report at the genetic level. Being the first of its kind in Barrett's adenocarcinoma, it is expected that other workers will confirm the result of this genetic study in future.

The topographic distribution of the abnormalities did not reveal any significant predilection for any part of the tumor except for a slightly more frequent occurrence of detectable genetic abnormalities at the periphery of the tumor compared to tumor center and from the superficial sites compared to the deeper sites . An objective analysis of this empirical observation is precluded by lack of comparable data in Barrett's adenocarcinoma, because no work of this nature has been carried out before on these tumors. The only study that is a little close is that from van Dekken and coworkers who studied chromosomal abnormalities in a multifocal Barrett's adenocarcinoma. Similar to our finding, they could not detect any significant difference in frequency of chromosomal anomalies between superficial and deeply situated tumor foci (van Dekken H et al., 1999). In contrast, Diaz-Cano et al. analyzed 44 urinary bladder transitional cell carcinomas (TCC) for intratumoral heterogeneity according to topography. They found a subset of tumors (38%) with topography related heterogeneity, in which the superficial tumor component showed predominant (neurofibromatosis-1) NF1-LOH, whereas the deeply situated invading tumor component showed p53-LOH in all cases tested (Diaz-Cano SJ et al., 2000). Since malignant transformation in BE is suspected to result from the action of some as yet undefined contents of the gastroduodenal refluxate and diet, it is possible that the superficial tumor region which is continuously bathed by these substances, might accumulate more genetic aberrations than the deep invading front, which is protected from such possible carcinogenic agents. This reason along with early onset of clonal diversity in Barrett's adenocarcinoma might be responsible for the failure to detect as much genetic aberrations in the metastases as in the corresponding tumor primaries. It could also partly explain the slightly more frequent occurrence of abnormalities from the superficial regions noted in this study. Perhaps, a larger number of cases could further elucidate this observation.

The frequency of genetic abnormalities detected in lymph node metastasis was low compared with the primary tumors as earlier mentioned. A simple reason for this could be the fact that only one lymph node site was investigated, whereas 8 different regions of the primary tumor were studied. Thus, there is simply a greater likelihood of detecting genetic anomalies from many sites within the primary tumor compared with only 1 single site of metastasis. However, the possibility remains that this finding might be a reflection of early onset of clonal diversity in Barrett's adenocarcinoma similar to findings in breast cancers (Fujii H et al., 1996; Kuukasjärvi T et al., 1997) and bladder cancers where clonal diversity was more pronounced in in situ lesions compared to accompanying invasive tumors. Alternatively, it might just be a reflection of the fact that metastasis occurs very early in Barrett's adenocarcinoma so that most of the aberrations detected in the primary

tumor occurred after the metastatic clone had migrated. Both of these propositions are, however, not mutually exclusive. Flow cytometry revealed that BE is a mosaic of cells and that progression in BE is associated with genetic instability (Rabinovitch PS et al., 1988). Galipeau et al. using a more specific microsatellite analysis technique was able to show a high degree of genetic heterogeneity in premalignant BE especially in the presence of HGD and concluded that neoplastic evolution in BE probably proceeds in a complex pattern with multiple early cell lineages rather than in a simple linear pathway (Galipeau PC et al., 1999). On the other hand, early metastasis is a notable feature of Barrett's cancers even when still at the intramucosal stage. This propensity for early tumor dissemination was empirically ascribed to the absence of an adventitial coat around the esophagus and the rich submucosal plexus of lymphatics. It is however not unlikely that this metastatic potential is due to the early evolution of a subclone with the genetic potential for tissue pene tration and invasion in Barrett's adenocarcinomas.

The detection of genetic abnormalities is the aim of so many research works on Barrett's adenocarcinoma. This usually involves the crude microdissection and collection of tumor cells from different parts of the tumor on the assumption that the tumor is clonal with respect to the investigated genetic abnormality. The demonstration of a high degree of genetic heterogeneity in Barrett's adenocarcinoma in this study has highlighted the potential error associated with such an assumption. Thus, mixture of different tumor areas could produce a dilutional effect where majority of tumor cells lack such an abnormality thereby preventing the detection of significant anomalies that may actually be present but within just a subclone of tumor cells. This could therefore lead to the false conclusion that such an abnormality is not at all present within the tumor. A phenomenon akin to the effect of contaminating normal stromal cells on PCR detection of specific mutant allele (Bianchi AB et al., 1991; Gruis NA et al., 1993). The technique of fractional microdissection employed in this study contributed to the high frequency with which certain genetic abnormalities were detected and is therefore strongly recommended in future research work on Barrett's adenocarcinoma.

Conclusion

The current work has confirmed that Barrett's adenocarcinoma is of monoclonal origin although with a high degree of clonal diversity and genetic heterogeneity in the tumor cell population. Moreover, the work also revealed specific oncogene and onco-suppressor gene abnormalities in Barrett's adenocarcinoma, thereby confirming and extending the findings of previous investigations. Of particular significance is LOH at the MCC gene and D4S1652 loci as well as amplification of the c-myc gene. Finally, despite the lack of significant topographic influence on the distribution of genetic abnormalities, the technique of fractional microdissection improved the detection of these abnormalities and is highly recommended in future research works on Barrett's cancers.

5. Summary

The intratumoral genetic heterogeneity is a poorly investigated aspect of molecular carcinogenesis, which is of high tumor biologic and therapeutic significance. The genetic heterogeneity in Barrett's adenocarcinoma was systematically investigated in the present work. Ten patients with esophageal adenocarcinomas who underwent surgical resection without radio- or chemotherapy provided the basis for the study. Following fixation in formalin and complete embedding of the resected specimens in paraffin, 8 primary tumor areas (tumor center and periphery and superficial and deep invasive tumor surfaces), a lymph node metastasis and an area of tumor free tissue from the resection margin were selected from each case for the following molecular biologic investigations. Extracted DNA from microdissected tissue was employed for PCR amplification of fragments of the tumor suppressor genes (TSG) APC, MCC, RB, D4S1652 and D18S474 and the proto-oncogene loci c-erbB-2, c-myc and EGFR. Finally, the PCR products from the various tumor areas were checked for the presence of allelic loss (LOH) with the aid of silver stained polyacrylamide gels. The detection of gene amplification in the 3 investigated proto-oncogenes was carried out with the aid of differential PCR, which included a control gene along with the investi gated gene.

From these investigations, there was LOH in 11% of informative cases in APC gene, in 57% in MCC gene, in 50% in RB gene, in 75% at the D4S1652 locus and in 0% at the D18S474 locus. Gene amplification was commonest in c-myc gene (33%), whereas it was less frequent in c-erbB-2 and EGFR genes, each with 10% rate. With regards to intratumoral genetic heterogeneity, it could be shown that in 94% of all cases with genetic aberrations (LOH and gene amplification), only some parts of the affected tumors were affected, whereas the remaining areas showed a wild type gene status. Concerning topographic distribution of genetic aberrations, however, no significant difference was detected between tumor center and periphery, and between superficial and deep invading tumor areas.

These results clearly show that genetic heterogeneity in Barrett's adenocarcinoma is a very frequent finding, which must be given serious consideration in future investigations of the molecular biologic abnormalities in this tumor type.

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7. Appendix

7.1 List of Abbreviations

AGE	Agarose gel electrophoresis
APC	Adenomatous polyposis coli
APRT	Adenosylphosphoribosyltransferase
BE	Barrett's esophagus
C-ERB	Cellular erythroblastomyelomatosis
C-MYC	Cellular myelocytomatosis
DCC	Deleted in colon cancer
DPC-4	Deleted in pancreatic carcinoma
dPCR	differential Polymerase chain reaction
EGFR	Epidermal growth factor receptor
GER	Gastroesophageal reflux
GES	Gastroesophageal sphincter
HGD	High grade dysplasia
IFN	Interferon
LES	Lower esophageal sphincter
LGD	Low grade dysplasia
LOH	Loss of heterozygosity
MCC	Mutated in colon cancer
MDC	Metaplasia-dysplasia-carcinoma
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
RB	Retinoblastoma
TGF	Transforming growth factor

7.2 Appreciation

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7.3 Curriculum Vitae

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1974 - 1980	Primary School, Iseyin, Nigeria
1980 - 1985	Grammar School, Iseyin, Nigeria
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1989 - 1990	Anatomy Demonstrator, Dept of Anatomy, Obafemi Awolowo University, Ile-Ife, Nigeria
1991 - 1992	Pre-registration House Officer
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1993 - 1994	Medical Officer, Remilekun Medical Centre, Lagos, Nigeria
1995 - 1998	Residency Training in Histopathology, Lagos University Teaching Hospital, Lagos, Nigeria
1998 till date	Institute of Pathology, Heinrich-Heine-University, Düsseldorf, Germany

Academic awards

1988	Best Student in the 1st Professional Exam
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1991	Student of the Year Award
1992	Best House Officer Award

Taofeek Kunle Owonikoko