The Journal of Laryngology & Otology
August 2001, Vol. **115,** pp. 639–644

The importance of dedifferentiation in recurrent acinic cell carcinoma

Conrad I. Timon, M.D., Irving Dardick, M.D.*

Abstract
The biological activity of acinic cell carcinoma is uncertain. Histological dedifferentiation is one possible reason for recurrent disease, and this study was undertaken to assess its importance in acinic cell carcinoma. The initial and recurrent specimens from five patients with acinic cell carcinoma were assessed histologically and using flow cytometry, AgNOR estimation and morphometric analysis for evidence of dedifferentiation. No objective evidence of a change in biological aggressiveness in recurrent acinic cell carcinoma was identified. In this limited series of a rare salivary gland tumour, it would appear that factors other than dedifferentiation, such as close/involved margins, histological type and stage have a more meaningful effect on the likelihood of recurrence and prognosis.

Key words: Carcinoma, Acinic Cell; Neoplasm Recurrence, Local; Tumour, Differentiation

Introduction

Acinic cell carcinoma is a rare, low-grade malignant salivary gland tumour with variable biological activity. Despite recent better understanding of the natural history of acinic cell carcinoma, the development of recurrence and metastases is unpredictable.[1,2] One theory for recurrence and metastases in acinic cell carcinoma is dedifferentiation, a well recognized behaviour in other tumour types.[3–5] Stanley *et al*.[6] described six salivary gland tumours containing areas of dedifferentiation with low-grade acinic cell carcinoma. In one patient, the dedifferentiation only became evident following analysis of the recurrent tumour tissue. These changes were based solely on histological features.[6]

Recently, a number of innovative techniques have been developed to examine cancer biology, and several of these techniques, in particular flow cytometry, nucleolar organizer region estimation and morphometry, allow the assessment of archival tumour material with the inherent advantage of instantaneous follow-up.

The objective of this study was to determine whether recurrent acinic cell carcinoma showed objective evidence of dedifferentiation based on ploidy, S phase, AgNOR estimation and morphometric analysis of the primary and recurrent tumour tissues.

Materials and methods

Patients with an original diagnosis of acinic cell carcinoma were identified by examining the files of the Toronto General Hospital, Toronto, the Head and Neck tumour registry in St Michael's Hospital, Toronto, and the Canadian Reference Centre for Cancer Pathology, Ottawa, Ontario. A total of 43 cases of histologically confirmed acinic cell carcinoma according to those established in previous reports were identified.[7,8] Of these, five cases were selected for study as they fulfilled the following criteria: histological and cytological criteria for the diagnosis of acinic cell carcinoma confirmed by the senior author, tumour tissue of initial and recurrent tumour material available for analysis, and clinical follow-up information available.

Microscopic examination

Paraffin blocks of formalin-fixed tissue from primary neoplasms and recurrences were stained with both haematoxylin and eosin and periodic acid Schiff (PAS) reaction (after diastase). For each neoplasm, the predominant cell type and architecture were recorded, as described by Abrams *et al*.[8] The pattern of growth and invasion and the presence or absence of lymphocytic infiltrate were also noted. An assessment of differentiation was recorded based on cell and nuclear shape and size, together with the number of mitotic figures, and each specimen was designated as either well-, moderately or poorly differentiated. Tumour tissue specimens stained with PAS (after diastase) were assessed and subjectively graded into no staining, moderate or extensive staining of mucin and extracellular materials.

From the Department of Otolaryngology, Head and Neck Surgery, St James's Hospital, Dublin, Ireland and the Department of Pathology*, The Toronto Hospital, Toronto, Canada.
Accepted for publication: 28 March 2001.

Flow cytometry

Nuclear suspensions were prepared using a modification of the methods of Hedley *et al.*[9] Briefly, three × 50 micron-thick sections were cut from each block. The sections were deparaffinized and a monodispersed cell suspension was obtained as follows: the tissues were mechanically broken up before enzymatic digestion using 0.5 per cent pepsin (Sigma Cat. #P-6887) in 0.1 N HCl (pH 1.2–1.5) at 37°C for a maximum of 1 hr with mixing every 10 min on a Vortex mixer.

Propidium iodide (PI; 3,8 diamino-5-diethyl-methyl-amino-propyl-6-phenylphenantridium diiodide; Sigma Cat. #P-4170) was used as a DNA dye in a 0.1 per cent hypotonic citrate solution.

Cytocentrifuge preparations of all nuclear suspensions were stained with May–Grunwal–Giemsa and microscopically checked for nuclear damage. A Becton Dickinson FACScan with a 15 mW, 488 nm argon-ion laser was used for analysis. DNA data acquisition was done after flow cytometric calibration with 1.75 micron CV beads (Polysciences Fluoresbrite beads, Cat. #18604) and linearity checks using chicken erythrocyte nuclei (Becton Dickinson). 10 000 events were collected for each sample in a LIST mode.

Histogram interpretation

Data analysis was carried out using Becton Dickinson CellFIT software version 1.2 with a doublet discrimination module (DDM). The histograms were classified without knowledge of survival or clinicopathological data. All cases with CV higher than eight per cent were omitted from calculations. The S phase percentage was determined by the CellFIT version 1.2 (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

AgNOR staining technique

A modification of the technique by Ploton *et al.*[10] was used for AgNOR staining. Four-micron-thick specimens were rehydrated, and the AgNOR staining solution obtained by mixing aqueous silver nitrate solution with gelatin solution in a 2 to 1 proportion by volume.

Each tissue section was covered by the AgNOR staining solution at room temperature in darkroom conditions for 50 min, then emersed in five per cent sodium thiosulphate for five min. This was followed by dehydration, xylene emersion and mounting. The AgNOR staining solutions was freshly prepared for every run.

The AgNOR slides were examined under oil immersion lens (magnification × 1000) with a green filter. Random fields were selected for counting; a simple eye piece graticule prevented recounting. Two hundred nuclei were studied from each tissue. The number of nuclei to be measured was determined by the principle of summation averages.

Morphometry

Thirty-four separate random fields of the AgNOR-stained acinic cell carcinoma slide were photographed in non-overlapping frames of high resolution 35-mm Kodak Tmax film (36 black and white) with a green filter. Following film development, each negative was projected onto a electronic digitizing pad (GITCO Corporation, Rockport, MD, USA) at a final magnification of 3500. The digitizing tablet was interfaced with a 40 MB hard drive IBM compatible PC using a software image analysis program (BQ System IV, R&M Biometrics). Only nuclear and AgNOR profiles that were sharply and completely outlined in each negative photographic frame were traced using a mouse; the digitized information was used to calculated the area, perimeter and form factor of each nuclear profile. In addition, the combined area and perimeters of the AgNORs present in each nucleus were measured together with the associated AgNOR count. Two hundred individual nuclei for each tumour were measured in this fashion.

Controls

Pleomorphic adenomas (three cases), myoepithelioma (one case) and associated normal salivary gland tissue were used as benign controls, while proven metastatic adenoid cystic (two cases) and adenocarcinomas (one case) were used as malignant controls.

Results

Clinical features

In five cases with 43 confirmed acinic cell carcinomas (patients one to five), it was possible to obtain between two and five (A–E Tables I–III) recurrent acinic cell carcinoma specimens for chronological comparison.

TABLE I
RECURRENCE OF DISEASE

Patient	Site (month)	Treatment	Second rec.	Treatment	Outcome	Follow-up (month)
1	Local (24)	Surgery	No	–	Alive	48
2	Local (96)	Surgery	Local × 2	Surgery	A with D	164
3	Local (24)	Surgery	Skull	Surgery/rad.	D of D	99
4	Reg. (09)	Neck diss.	Reg. × 2	Surgery	A with D	144
5	Skull base (66)	Chemo.	No	–	D of D	80

rec. = recurrence, reg. = regional recurrence, chemo. = chemotherapy, rad. = radiotherapy, D of D = dead of disease, A with D = alive with disease.

TABLE II
RECURRENT ACINIC CELL CARCINOMA (PATHOLOGY)

Patient	Year	Arch.	Cell type	Differentiation	PAS stain
1a	1986	Solid	Acinic	Well	Very positive
1b	1989	Solid	Acinic	Well	Very positive
2a	1976	Solid	Acinic	Well	Very positive
2b	1985	Micro.	Acinic	Well	Very positive
2c	1986	Mixed	Acinic	Well	Very positive
2d	1988	Solid	Acinic	Well	Very positive
2e	1988	Mixed	Acinic	Well	Very positive
3a	1976	Mixed	Acinic	Well	Very positive
3b	1981	Solid	Acinic	Well	Very positive
3c	1982	Solid	Acinic	Well	Very positive
3d	1984	Solid	Acinic	Well	Very positive
4a	1978	Solid	Acinic	Well	Very positive
4b	1979	Papil.	Acinic	Well	Very positive
4c	1980	Papil.	Acinic	Well	Moderate
5a	1975	Papil.	Inter. duct	Poor	Moderate
5b	1981	Mixed	Inter. duct	Moderate	Negative

Arch. = architecture, Papil. = papillary type architecture, Inter. duct. = intercalated duct cell, PAS = periodic acid Schiff.

All five patients underwent surgery as an initial treatment (Table I). In patients one and five, initial local excision was followed by a parotidectomy between three weeks and five months after the first operation. The other three patients underwent parotidectomy at initial diagnosis. In addition, patients one and four underwent a neck dissection, and patients five underwent post-operative radiotherapy.

Patients 1, 2, 3 and 5 developed local recurrence between 24 and 96 months following diagnosis. Patient 4 developed cervical nodal metastases at 17 months. Apart from patient five, who underwent chemotherapy for local skull base unresectable disease following biopsy, the other four patients underwent surgery (Table I) for the management of recurrence.

Patients 3 and 5 ultimately died from disease 99 and 81 months respectively after the initial diagnosis. Patients 2 and 4 are alive with local disease recurrence 164 and 144 months, respectively, after initial presentation, while patient 1 is alive without disease 48 months after diagnosis.

Pathology

A comparison of the pathological findings are shown in Table II. This showed that, apart from predominant architecture, the histological parameters remained stable with recurrences. This was also true for other parameters not illustrated in Table II, such as lymphocytic infiltration, and adjacent infiltration or acinic cell carcinoma into normal salivary tissue. In patient 5, the differentiation was considered poor at first presentation and moderate on recurrence six years later. Interestingly, there was a corresponding change in PAS staining from moderate to negative over the same period.

The architecture showed a marked variation between initial tumour and recurrent tumour assessment; however, there was no clear-cut pattern. While in patient 2 the initial architecture was solid in type, changing to mixed on recurrence, the opposite was true for patient 3. Similarly, while the tumour in patient 4 changed from solid to papillary predominant upon recurrence, in patient 5 the papillary predominant architecture at initial presentation changed to mixed type on recurrence.

TABLE III
RECURRENT ACINIC CELL CARCINOMA (FLOW CYTOMETRY AND AGNOR COUNTS)

Patient	Year	Ploidy	S phase (per cent)	AgNOR count
1a	1986	Debris	–	3.07
1b	1989	Diploid	Low	2.10
2a	1976	Diploid	6.3	2.59
2b	1985	Diploid	5.4	1.82
2c	1986	Diploid	2.0	2.48
2d	1988	Diploid	1.1	1.61
2e	1988	Diploid	Low	2.15
3a	1976	Diploid	0.3	2.64
3b	1981	Aneuploid	0.8	2.64
3c	1982	Aneuploid	1.0	2.33
3d	1984	Aneuploid	Low	2.52
4a	1978	Diploid	1.0	1.89
4b	1979	Aneuploid	4.6	2.43
4c	1980	Debris	–	2.84
5a	1975	Aneuploid	1.1	1.58
5b	1981	Diploid	2.8	2.7

TABLE IV
RECURRENT ACINIC CELL CARCINOMA (MORPHOMETRY)

Patient	Year	Nuclear area (μm)	AnNOR area (μm)	Shape factor	AgNOR count
1a	1986	27.58	2.16	0.917	2.23
1b	1989	24.05	1.83	0.817	1.70
2a	1976	35.71	1.02	0.943	2.00
2b	1985	24.55	2.04	0.863	1.42
2c	1986	29.45	2.02	0.913	1.69
2d	1988	29.88	2.01	0.873	1.53
2e	1988	25.63	1.98	0.907	1.63
3a	1976	25.89	1.68	0.923	1.70
3b	1981	25.00	2.09	0.914	2.10
3c	1982	32.23	2.19	0.944	2.33
3d	1984	18.60	1.91	0.870	1.03
4a	1978	21.35	1.52	0.880	1.34
4b	1979	21.49	1.99	0.884	1.96
4c	1980	41.03	2.03	0.902	2.14
5a	1975	25.42	1.66	0.864	1.34
5b	1981	29.88	2.01	0.873	1.61

Overall, there was no evidence on histological assessment that dedifferentiation was a factor in the aetiology of recurrence, and there was no obvious relationship between chronological pathological changes and eventual outcome.

Flow cytometry

The results of flow cytometry are illustrated in Table III. Three patients (patients 2, 3 and 4) were considered diploid at initial presentation. Patient 5 was aneuploid, while it was not possible to assess patient one because of excessive debris. Patient 2 continued to be diploid on subsequent recurrences but the recurrent specimens for patients 3 and 4 changed to aneuploid. The reverse was true for patient 5, in which the initial aneuploid status changed to diploid with recurrence. S phase using the RFIT model showed no particular trend to increase with recurrence.

Nucleolar organizer regions

Table III illustrates the nucleolar organizer regions for each patient. An increasing chronological AgNOR count was present in patients 4 and 5 but absent in patients 2 and 3 and reversed for patient one. This was also true for AgNOR estimation using morphometry (Table IV).

Morphometry

Analysis of morphological parameters (Table IV) also fails to show a clear trend for recurrent disease. The nuclear area decreased with recurrence with an overall more irregularly shaped nucleus for patients 1, 2 and 3. However, the nuclear area and shape factor showed the opposite trend for patients 4 and 5. There was no correspondingly poorer outcome for the former three patients. Nor did the AgNOR area show a specific trend with recurrence.

Discussion

Acinic cell carcinoma is considered a low-grade salivary gland tumour, difficult to study because of its rarity and the relatively recent observation of its significant malignant potential.[11] Although present ubiquitous histological methods allow the diagnosis of acinic cell carcinoma to be made with some degree of certainty, they have unfortunately failed to provide a reliable indication of biological activity.[1,2,7] This has resulted in much controversy and ambivalence regarding therapeutic measures used in its treatment.[12] New techniques such as flow cytometry and nucleolar organizer region evaluation are increasingly being used with varying results in the evaluation of borderline lesions and low-grade malignancies. Abnormalities in cellular DNA content measured by flow cytometry have correlated well with histological differentiation, and to a certain extent clinical behaviour in a wide variety of tumours.[13,14] S phase determination has also shown promise as a prognostic parameters.[15] However, little information exists regarding its use in acinic cell carcinoma analysis, and what is available is contradictory.[16,17] Nucleolar organizer regions are recognized as loops of DNA which transcribe to ribosomal RNA, and can be located accurately by staining with silver nitrate under prescribed conditions, the structures thus demonstrated are termed 'AgNORs'.[18] The technique has been used both as an adjunct to differential diagnosis and for prognostication, with variable results.[17]

Morphometric analysis allows cellular and nuclear dimensions of tumours cells to be measured quantitatively and has provided important prognostic information in a number of lymphoreticular and solid tumour types.[19,20] There has been limited application of image analysis in diagnosis problems in salivary gland pathology. Layfield *et al.*[21] reported a significant increase in nuclear parameters in malignant cases. What makes these processes parti-

https://doi.org/10.1258/0022215011908711 Published online by Cambridge University Press

cularly attractive to study in acinic cell carcinoma is that archival tumour material with a known outcome may be investigated.

Dedifferentiation is widely accepted to occur in head and neck tumours such as those with a high proportion of anaplastic thyroid carcinomas arising from the milieu of a pre-existing well-differentiation thyroid carcinoma and the entity of carcinoma ex pleomorphic adenoma in which a carcinoma (usually an adenocarcinoma) develops from a pre-existing benign pleomorphic adenoma.[6] Stanley *et al.*, in 1988,[6] introduced the term 'dedifferentiated acinic cell carcinoma' to explain the occurrence of six salivary gland neoplasms containing areas of low-grade acinic cell carcinoma together with high-grade adenocarcinomas. They felt that their occurrence was evidence of dedifferentiation of acinic cell carcinoma, a process felt by Batsakis to be 'not probable'.[22] In one patient in Stanley *et al.*'s study, the dedifferentiation became apparent only after recurrence, although unlike our study, Stanley *et al.*[6] did not compare initial and recurrent acinic cell carcinoma specimens.

In an effort to see whether dedifferentiation may explain the recurrence rate in acinic cell carcinoma, a large series on these tumours was examined and samples selected where both the original and recurrent specimens were present. The progression of recurrence for acinic cell carcinoma was studied by means of routine histology, flow cytometry, S phase analysis morphometry and AgNOR determination.

In contrast to Stanley *et al.*'s[6] findings no histological evidence of dedifferentiation was apparent in the five cases of acinic cell carcinoma with multiple specimens available. A previous paper by Timon *et al.*[23] suggested that the solid acinic cell carcinoma histological pattern had a poorer prognosis than the microcystic type; however, although architecture varied widely between recurrent specimens in this study there was no uniform pattern to suggest a direct correlation.

Flow cytometry analysis of these recurrent acinic cell carcinomas also showed a varying picture. In two patients, an initial diploid state at presentation changed to aneuploidy with recurrence. There was a corresponding moderate increase in S phase percentage. These results could theoretically be put forward as evidence of acinic cell carcinoma dedifferentiation.

Another possible reason for this change in ploidy, and a more likely explanation, is that the aneuploid tumour tissue was missed because of cellular heterogeneity. Thus, information obtained from a single area of the tumour may not necessarily be representative of the entire specimen. This possibility is supported by Wersto *et al.*[24] who found that flow cytometry of single specimens sampling containing colorectal adenocarcinomas may miss aneuploid subpopulations in 15–20 per cent of cases. Further supporting this possibility in this study is the fact that one patient who developed a recurrence was initially aneuploid but the recurrence was diploid.

Similarly, no clear pattern emerged between progression of disease and morphometry or AgNOR parameters. It is possible that, although complex, these techniques are not sensitive enough at present to identify a change in biological behaviour in tumour specimens. The small number of patients studied in this paper, (only five of the initial 43 met the inclusion criteria) is a potential weakness as far as the conclusions are concerned. Further larger studies are warranted but may be difficult to carry out because of the rarity of this tumour type and the difficulty in obtaining pathological specimens of recurrent tumour.

In summary, in-depth analysis of recurrent acinic cell carcinoma specimens failed to show convincing evidence of a change in biological aggressiveness. It would appear that other factors such as close/involved margins, histological type and stage may have a more meaningful effect on the likelihood of recurrence and prognosis.

References

1 Ellis GL, Corio RL. Acinic cell adenocarcinoma. *Cancer* 1983;**52**:542–9
2 Lewis JE, Olsen KD, Weiland LH. Acinic cell carcinoma: clinicopathologic review. *Cancer* 1991;**67**:172–9
3 Evans HL. Liposarcoma: a study of 55 cases with a reassessment of its classification. *Am J Surg Pathol* 1979;**3**:507–23
4 Wold LE, Unni KK, Beabout JW, Sim FH, Dahlin DC. Dedifferentiated parosteal osteosarcoma. *J Bone Joint Surg* 1984;**66**:53–9
5 Dahlin DC, Beabout JW. Dedifferentiation of low-grade chondrosarcoma. *Cancer* 1971;**8**:461–6
6 Stanley RJ, Weiland LH, Olsen KD, Pearson BW. Dedifferentiated acinic cell (acinous) carcinoma of the parotid gland. *Otolaryngol Head Neck Surg* 1988;**98**:155–61
7 Eneroth CM, Jakobsson PA, Blanck C. Acinic cell carcinoma of the parotid gland. *Cancer* 1966;**19**:1761–72
8 Abrams AM, Cornyn J, Scofield HH, Hansen LS. Acinic cell adenocarcinoma of the major salivary glands. *Cancer* 1965;**18**:1145–62
9 Hedley DW, Friedlander ML, Taylor LW, Rugg CA, Musgrove EA. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem* 1983;**31**:1333–5
10 Ploton D, Menager M, JeannessonP, Himber G, Pigeon F, Adnett JJ. Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organiser regions at the optical level. *Histochem J* 1986;**18**:5–15
11 Buxton RW, Maxwell JH, French AJ. Surgical treatment of epithelial tumours of the parotid gland. *Surg Gynaecol Obst* 1953;**97**:401–16
12 Perzin KH, LiVolsi VA. Acinic cell carcinoma arising in salivary glands. *Cancer* 1979;**44**:1434–57
13 Lundberg S, Carstensen J, Rundguist I. DNA flow cytometry and histopathological grading of paraffin-embedded prostate biopsy specimens in a survival study. *Cancer Res* 1987;**47**:1973–7
14 Klemi PJ, Joensuu H, Salmi T. Prognostic value of flow cytometric DNA content analysis in granulosa cell tumour of the ovary. *Cancer* 1990;**65**:1189–93
15 Fisher B, Gunduz N, Costantino J, Fisher ER, Redmond C, Mamounas EP, *et al.* DNA flow cytometric analysis of primary operable breast cancer. *Cancer*1991;**67**:1465–75
16 El-Nagger AK, Batsakis JG, Luna MA, McLemore D, Byers RM. DNA flow cytometry of acinic cell carcinomas of major salivary glands. *J Laryngol Otol* 1990;**104**:410–16

17 Timon CI, Dardick I, Panzarella T, Thomas T, Ellis E, Gullane P. Acinic cell carcinoma of salivary glands: the prognostic relevance of DNA flow cytometry and nucleolar organiser regions. *Arch Otolaryngol Head Neck Surg* 1994;**120**:727–33
18 Morgan DW, Crocker J, Watts A, Shenoi PM. Salivary gland tumours studied by means of the AgNOR technique. *Histopathology* 1988;**13**:553–9
19 Lipponen P, Simpanen H, Pesonen E, Esskelinen M, Sotarauta M, Collan Y. Potential of morphometry in grading transitional cell carcinoma of the urinary bladder. *Pathol Res Pract* 1989;**185**:617–20
20 Tosi P, Baak JPA, Miracco C, Vindigni C, Lio R, Barbini P. Gastric dysplasia: a stereological and morphometrical assessment. *J Pathol* 1987;**152**:83–94
21 Layfield LJ, Hall TL, Fu YS. Discrimination of benign versus malignant mixed tumours of the salivary gland using digital image analysis. *Cytometry* 1989;**10**:217–21
22 Batsakis JG (ed). *Tumors of the Head and Neck: Clinical and Pathological Considerations*, 2nd Edn. Baltimore: Williams and Wilkins, 1979:39–44
23 Timon CI, Dardick I, Panzarella T, Thomas J, Ellis E, Gullane P. Clinico-pathological predictors of recurrence for acinic cell carcinoma. *Clin Otolaryngol* 1995;**20**:396–401
24 Wersto RP, Liblit RL, Deitch D, Koss LG. Variability in DNA measurements in multiple tumor samples of human colonic carcinomas. *Cancer* 1991;**67**:106–15

Address for correspondence:
Professor C. I. Timon,
Department of Otolaryngology, Head and Neck Surgery,
St James's Hospital,
James's Street,
Dublin 8,
Ireland.

Professor C. Timon takes responsibility for the integrity of the content of the paper.
Competing interests None declared