ELSEVIER

European Journal of Cancer 40 (2004) 503–507

European Journal of Cancer

www.ejconline.com

Prognostic value of facilitative glucose transporter Glut-1 in oral squamous cell carcinomas treated by surgical resection: results of EORTC Translational Research Fund studies

R.J. Oliver[a,*], R.T.M. Woodwards[b], P. Sloan[a], N.S. Thakker[a], I.J. Stratford[c], R.E. Airley[d]

[a]*Oral and Maxillofacial Sciences, University Dental Hospital of Manchester, Higher Cambridge Street, Manchester M15 6FH, UK*
[b]*North Manchester General Hospital, Crumpsall, Manchester, UK*
[c]*School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, UK*
[d]*School of Pharmacy, Liverpool John Moores University, Liverpool, UK*

Received 27 May 2003; received in revised form 30 August 2003; accepted 28 October 2003

Abstract

Hypoxia in tumours of the oral cavity has not been extensively investigated with regard to clinical outcome and prognosis. The expression of the facilitative glucose transporter, Glut-1, has been shown to be related to hypoxia in tumours at other sites. The aim of the present study was to investigate the relationship between Glut-1 expression and clinical outcome in a series of oral squamous cell carcinomas. A retrospective series of 54 cases of oral squamous cell carcinomas with known clinical outcome and treated by one surgeon over a period of 6 years was used in the study. A representative section from each case was stained immunohistochemically with an antibody against Glut-1. The stained sections were then assessed independently by two observers using a semi-quantitative method. The relationship between these results and the clinical outcomes of local recurrence, regional lymph-node metastasis and disease-free survival were examined. Glut-1 staining was observed in most of the tissue specimens and all of the few sections with demonstrably necrotic areas histologically. Some showed more prominent staining in the epithelial islands of the tumour than others. However, the intensity of staining was variable. There was a significant relationship between those tumours which demonstrated intense staining and recurrence overall ($\chi^2 = 6.18$, $P = 0.032$). This relationship was strongest in relation to regional lymph-node recurrence ($\chi^2 = 10.19$, $P = 0.005$). A significant relationship between disease-related death and intense Glut-1 staining was also observed ($\chi^2 = 11.67$, $P = 0.002$). In conclusion, the results of this study indicate a relationship between Glut-1 expression and disease progression of oral cancer and could indicate a need for neoadjuvant chemoradiotherapy for those tumours demonstrating intense Glut-1 expression.

Keywords: Cell hypoxia; Glut-1 protein; Glucose transport protein; Hypoxia; Mouth neoplasms; Oral cancer; Prognosis

1. Introduction

Oral squamous cell carcinoma is estimated to be the 12th commonest cancer worldwide [1], with significant regional variation in incidence and evidence, In some countries, such as the United Kingdom (UK) for example, the incidence is once again increasing [2,3]. The primary treatment for this disease in most cases is surgery with radiotherapy being used as an adjunct to surgery or when the tumours are inoperable. Despite advances in surgical techniques over the past 20 years, the overall 5-year survival remains poor, in the region of 50%, although smaller tumours have a better prognosis [4]. Local or regional lymph node recurrence accounts for most treatment failures [4].

Malignant cells operate at an increased rate of respiration, glucose uptake and glycolysis [5]. This upregulation of glycolytic metabolism helps maintain the increased energy requirements of rapidly proliferating cells, and represents an important adaptive change

* Corresponding author. Tel.: +44-161-275-6624/50; fax: +44-161-275-6631.
E-mail address: richard.j.oliver@man.ac.uk (R.J. Oliver).

0959-8049/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2003.10.024

believed to be necessary to overcome the adverse microenvironmental conditions existing in tumours. For this reason, these metabolic changes have traditionally been used to provide diagnostic and prognostic information, for example in the use of fluorodeoxyglucose-positron emission tomography (FDG-PET), where increased FDG uptake can be used to detect malignancies and as an indicator of poor prognosis [5–7]. In tumours, increased glucose uptake is chiefly achieved through the upregulation of several of a large family of facilitative glucose transporters (gluts). In particular, the facilitative glucose transporter, Glut-1, is overexpressed in a wide variety of tumours, and analogous to FDG uptake, predicts poor a prognosis [8,9]. An important environmental factor in the control of glucose transporter expression is tumour hypoxia.

It is well established that tumour hypoxia leads to radioresistance [10], increased malignancy and an increased likelihood of metastasis [11]. In addition, it is also thought that hypoxia is a determining factor in the response to most conventional chemotherapies. This is due, in part, to their oxygen dependent cytotoxicity, but also to the inherent chemoresistance occurring as a consequence of the cell-cycle changes and poor vascularisation observed in hypoxic tumours [12]. A significant number of studies have investigated the presence of hypoxia in head and neck cancer, where hypoxia has been shown to be an indicator of poor prognosis in tumours of the head and neck treated with radiotherapy [13–15]. Finally, the more aggressive 'hypoxic phenotype' arises due to the stimulation of a variety of hypoxia-regulated genes, which act as putative survival signals and allow the tumour to adapt and flourish in this adverse environment [11].

These hypoxia-regulated genes, of which Glut-1 is an example, are expressed via the HIF-1 transcription factor. There is significant clinical evidence that tumour hypoxia may lead to poor outcomes by mediating such immunohistochemically-detectable genetic changes. Recently, the expression of hypoxia-regulated genes, such as Glut-1 [16] and carbonic anhydrase-9 (CaIX) [17], as intrinsic markers of tumour hypoxia were investigated in advanced carcinoma of the cervix and compared with direct oxygen measurements and with the binding of the bioreductive drug marker of hypoxia, pimonidazole [16]. The correlation between Glut-1 and CaIX expression and pimonidazole binding was particularly strong. However, in contrast to measurements of overall tumour pO_2 which correlated with outcome of treatment as assessed by local tumour control, measurements of both Glut-1 and CaIX predicted for metastasis-free survival, but not local control. This suggests that the expression of the proteins encoded by these hypoxic-regulated genes may well be strong indicators of recurrence and distant spread of disease. To test the generality of this hypothesis, we have taken a series of squamous cell carcinomas of the oral cavity treated with surgery as the primary modality, to examine the clinical significance of the expression of Glut-1 as a potential prognostic indicator.

2. Patients and methods

2.1. Patients

A retrospective series of 54 cases of primary oral squamous cell carcinomas with a known clinical outcome and treated curatively by one surgeon at the North Manchester General Hospital over a period of 6 years was used in this study. The cases were from the following sites: tongue (21/54, 39%), floor of mouth (16/54, 30%), retromolar trigone (8/54, 15%), gingiva/alveolus (3/54, 6%), maxilla (3/54, 6%), buccal (3/54, 6%). Clinical data regarding recurrence locally, regionally and overall survival were available for a minimum of 5 years for all of the patients. Local research ethics committee approval was sought and obtained (North Manchester LREC study number 02/104). A representative radial tissue block was selected for each case based upon viewing of the haematoxylin and eosin stained sections histologically. Four micron sections were cut and mounted on silane-coated microscope slides for the immunohistochemical staining below.

2.2. Immunohistochemical staining for Glut-1

Immunohistochemical staining for Glut-1 expression was carried out using a standard immunoperoxidase technique which is described briefly as follows. Sections were dewaxed in xylene and rehydrated through decreasing concentrations of ethanol to water. Staining was performed using an Envision kit containing an anti-rabbit labelled polymer conjugate (DAKO, UK). The primary antibody used was an affinity purified anti-rabbit Glut-1 (dilution: 1/100) (Alpha Diagnostic International, TX, USA). A rabbit IgG (10 μg/ml, DAKO, UK) served as a negative control. Glut-1 expression is prolific in erythrocytes, therefore, the presence of these cells within tumour capillaries in the section provided an internal positive control. Two batch controls were included in each run.

2.3. Semi-quantitative analysis

A scoring system previously described in Ref. [16] was used. Briefly, stained sections were viewed at an objective magnification of ×10 and a score given according to the intensity of Glut-1 staining within the tumour. The following scores were 0, no staining; 1, light staining; 2, moderate staining; 3 heavy/intense staining. The

sections were viewed independently by two observers and consensus scores reached in cases of discrepant scores. Additionally, the staining pattern within the epithelium of the tumour was noted as predominantly basal, suprabasal or both. The adjacent epithelium which also represented the surgical margin was scored according the semi-quantitative system outlined above.

The results of the above were compared with the clinical data using the χ^2 test and Kaplan–Meier survival statistics and curves. The following clinical outcomes were examined, recurrence at the primary site (local), recurrence in the regional lymph nodes and overall disease-free survival.

3. Results

Of the 54 cases, 36 were male (mean age 65 years) and 18 were female patients (mean age 67 years), with an overall mean age of 65 years (standard deviation (S.D.) 13.1, range 35–89 years). There were eight cases with local recurrence and 11 cases with regional recurrence. 22 of the 54 cases died, but only 10 died of their disease.

Some degree of Glut-1 staining was observed in all, but one, of the sections. Where histologically-evident areas of necrosis were present, intense Glut-1 staining was evident, as would be expected (Fig. 1). A range of staining patterns were observed within the epithelia; some showed a predominantly basal staining pattern with the superficial layers showing little or no Glut-1 staining (Fig. 2). Other sections showed staining predominantly in the superficial layers of the epithelia (Fig. 1). These staining patterns did not relate to clinical outcome in any way.

Of the staining intensities in the tumours, 13 cases were graded as staining intensely, 16 cases as moderate, 20 as light and 5 as negative. Initially, two intensity groups were examined comprising no staining and light staining as group one and moderate and intense staining as group 2. Using the χ^2 test, these two groups were compared with the outcomes of local and regional recurrence, recurrence overall and death due to disease. None of these reached significance. However, when the groups were stratified so that one group comprised only intense Glut-1 staining and the other group comprised all of the other cases, some significant relationships were observed. There was a significant relationship between intense Glut-1 staining and recurrence overall ($\chi^2 = 6.18$, $P = 0.032$, Fig. 3) which was accounted for by the strong association between intense Glut-1 staining and regional recurrence ($\chi^2 = 10.19$, $P = 0.005$), as there was no significant relationship with local recurrence ($\chi^2 = 1.88$, $P = 0.355$). There was also a significant association between death due to disease and intense Glut-1 staining ($\chi^2 = 11.67$, $P = 0.002$), but there was no association between Glut-1 staining and overall survival. The results are summarised in Table 1. 13 cases received post-operative radiotherapy, this did not affect the overall survival or the relationship of Glut-1 staining intensity with outcome. A strong correlation between observers was noted with a kappa value of 0.767 and Spearman rank coefficient of 0.935 ($P < 0.001$).

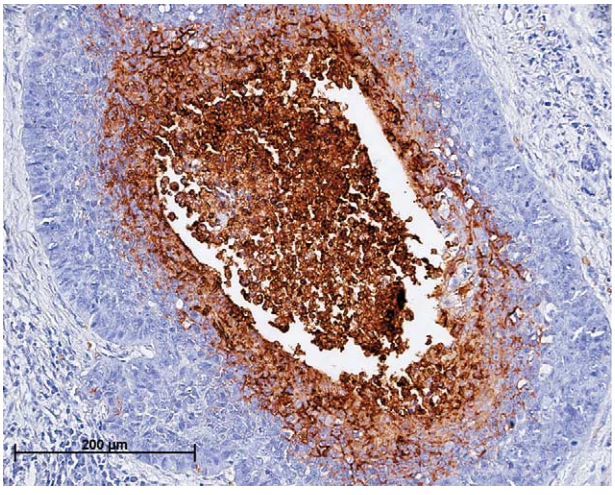

Fig. 1. Island of squamous cell carcinoma with central necrosis demonstrating intense Glut-1 staining and lack of basal epithelial staining.

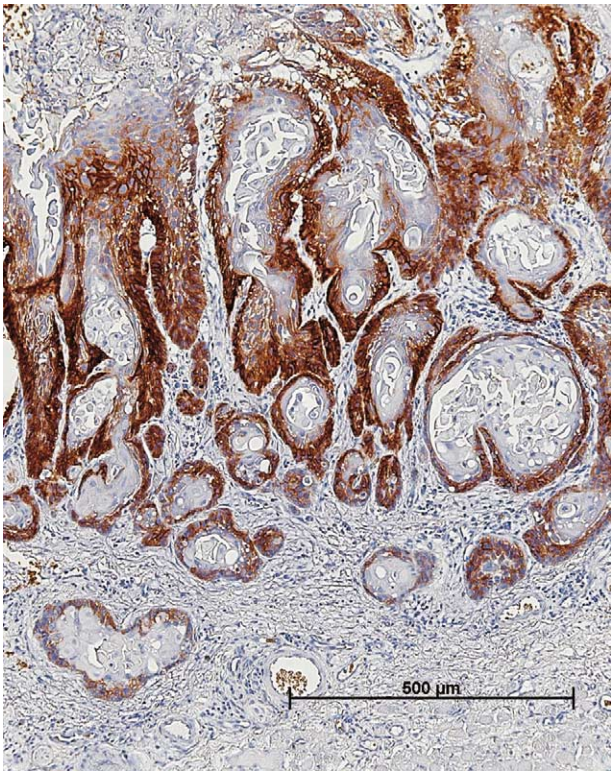

Fig. 2. Demonstrating predominantly basal Glut-1 staining within the epithelium.

Table 1
Summary of the results

Glut-1 Intensity	*n*	Local recurrence	Regional recurrence	Recurrence (local or regional)	Disease-related death
0 (no staining)	5	1	0	1	0
1 (light staining)	20	2	1	3	0
2 (moderate staining)	16	2	2	4	2
3 (intense staining)	13	3	6*	9**	8***

*$\chi^2 = 10.19$, $P = 0.005$; **$\chi^2 = 6.18$, $P = 0.032$; ***$\chi^2 = 11.67$, $P = 0.002$.

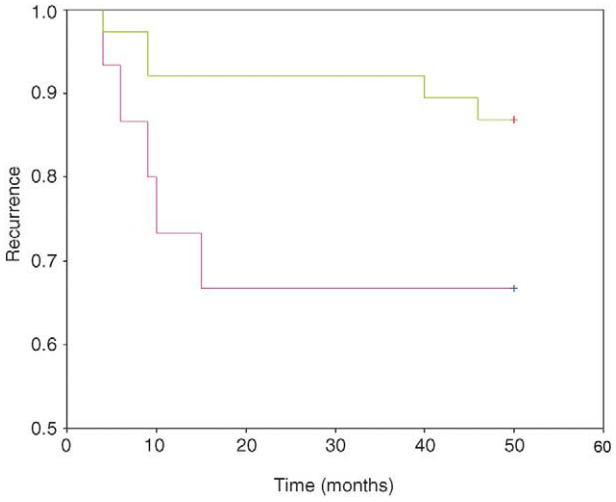

Fig. 3. Kaplan–Meier curve of Glut-1 staining and recurrence overall (weak Glut-1 staining in green, strong Glut-1-positive in pink).

4. Discussion

In the present study, we have demonstrated a relationship between intense Glut-1 staining and both regional metastasis and disease-related death in a series of oral cancers treated with surgery as their primary treatment.

In cancers of the head and neck treated with chemoradiotherapy, previous investigations have shown that the overexpression of HIF-1-regulated genes [18] are indicators of poor prognosis. However, the role of Glut-1 is not known. Glut-1 is invariably overexpressed in head and neck tumours, but a recent study in a series of patients with laryngeal carcinoma found that although the HIF-1-regulated Glut-3 predicted a poor outcome, the consistent overexpression of Glut-1 in associated squamous epithelial layers meant that Glut-1 was ineffective as a prognostic indicator [19]. However, the work reported here shows that, in tumours of the oral cavity, Glut-1 immunostaining has two distinct patterns. Where Glut-1 staining occurs in outer epithelial layers, this may be a function of differentiation and invasion processes [20], whereas Glut-1 staining existing in central tumour nests, distal to blood vessels, may signify the existence of hypoxia-induced Glut-1. Although similar patterns of expression of Glut-1 to those observed in the present study were reported by Reissner and colleagues [20] in head and neck cancer, these authors did not correlate this with prognosis. Recently, Kunkel and colleagues [21] reported that oral tumours with a high Glut-1 labelling index had a poorer overall survival, but did not state if this was disease-related death or if there was any correlation with loco-regional disease control.

A previous similar study in the uterine cervix also demonstrated a relationship between Glut-1 expression and metastasis-free survival [16]. In addition, in surgically-treated oesophageal cancers, Glut-1-positive tumours were shown to have a poorer survival [22]. Brizel and colleagues [23] recently reported elevated tumour lactate concentrations in head and neck cancers (mostly of the larynx) that were associated with metastasis and disease-free survival with most of the patients being treated primarily with radiotherapy. These authors suggest this may be due to a predominantly glycolytic metabolism in these tumours due to hypoxia. CaIX, one of a group of proteins involved in the hydration of carbon dioxide to carbonic acid, has been used as another surrogate marker for hypoxia since it is upregulated by hypoxia. Strong CaIX expression has been demonstrated in head and neck tumours in areas of poor vascularisation and significantly reduced the response to chemoradiotherapy [18]. Using a polarographic electrode to assess tumour oxygenation, Brizel and colleagues [14] reported that hypoxia affected the three year regional control, disease-free survival in a series of head and neck cancers (mostly of the oropharynx) treated primarily with radiotherapy. Additionally, they demonstrated that poorly oxygenated lymph nodes were more likely to contain residual tumour in the post-irradiation neck dissection.

The results of our study and other similar studies using surrogate markers for tumour hypoxia have implications for the management of the tumours. Phase three trials are currently underway of a novel radiotherapy approach known as accelerated radiotherapy with carbogen and nicotinamide (ARCON) [24,25]. This method uses hyperoxic gas (carbogen) in combination with a vasoactive agent (nicotinamide) in an attempt to decrease tumour hypoxia and increase the response to radiotherapy; studies so far have demon-

strated a better disease-free control in the larynx and oropharynx, but disappointing results in the oral cavity [25]. This is probably because hypoxia is not important for local control of the disease, but our results show that expression of Glut-1 (as a potential marker of hypoxia) is an indicator of progression. Similarly, hypoxia has been demonstrated to be related to progression of disease in carcinoma of the cervix, irrespective of the primary treatment modality [26].

In conclusion, the results of the present study suggest that Glut-1 expression in oral cancers could indicate the need for neoadjuvant chemoradiotherapy. In the future gene therapy targeted at Glut-1 [27] following surgical resection could be used regardless of completeness of resection and extracapsular lymph-node metastases. This could be used as is presently the case in the UK.

Acknowledgements

We would like to acknowledge Mrs Sue Holland for the preparation of the sections. This work was supported by grants from the Medical Research Council and the European Organisation for Research and Treatment of Cancer (EORTC) TRF grants (TRF/01/03 to I.J.S. (Screening and Pharmacology Group) and TRF/01/04 to P.S. (Head and Neck Group).

References

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999, **80**, 827–841.
2. Hindle I, Downer MC, Speight PM. The epidemiology of oral cancer. *Br J Oral Maxillofac Surg* 1996, **34**, 471–476.
3. Robinson KL, Macfarlane GJ. Oropharyngeal cancer incidence and mortality in Scotland: are rate still increasing? *Oral Oncol* 2003, **39**, 31–36.
4. Haddadin KJ, Soutar DS, Webster MHC, Robertson AG, Oliver RJ, MacDonald DG. Natural history and patterns of recurrence of tongue tumours. *Br J Plast Surg* 2000, **53**, 279–285.
5. Worburg O. *The Metabolism of Tumours*. London, Constable, 1930.
6. Minn H, Lapela M, Klemi PJ, *et al*. Prediction of survival with fluorine-18-fluorodeoxyglucose and PET in head and neck cancer. *J Nucl Med* 1997, **38**, 1907–1911.
7. Nakasone Y, Inoue T, Oriuchi N, *et al*. The role of whole-body FDG-PET in preoperative assessment of tumor staging in oral cancers. *Ann Nucl Med* 2001, **15**, 505–512.
8. Kawamura T, Kusakabe T, Sugino T, *et al*. Expression of glucose transporter-1 in human gastric carcinoma—association with tumor aggressiveness, metastasis, and patient survival. *Cancer* 2001, **92**, 634–641.
9. Rege S, Safa AA, Chaiken L, Hoh C, Juillard G, Withers HR. Positron emission tomography: an independent indicator of radiocurability in head and neck carcinomas. *Am J Clin Oncol* 2000, **23**, 164–169.
10. Nordsmark M, Overgaard M, Overgaard J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. *Radiother Oncol* 1996, **41**, 31–39.
11. Nordsmark M, Overgaard J. A confirmatory prognostic study on oxygenation status and loco-regional control in advanced head and neck squamous cell carcinoma treated by radiation therapy. *Radiother Oncol* 2000, **57**, 39–43.
12. Koukourakis MI, Giatromanolaki A, Sivridis E, *et al*. Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. *Int J Radiat Oncol Biol Phys* 2002, **53**, 1192–1202.
13. Behrooz A, Ismail-Beigi F. Stimulation of glucose transport by hypoxia: signals and mechanisms. *News Physiol Sci* 1999, **14**, 105–110.
14. Brizel DM, Dodge RK, Clough RW, Dewhirst MW. Oxygenation of head and neck cancer: changes during radiotherpy and impact on treatment outcome. *Radiother Oncol* 1999, **53**, 113–117.
15. Nordsmark M, Overgaard M, Overgaard J. Testing the hypothesis: pretreatment oxygenation predicts radiation response in advanced head & neck squamous cell carcinoma. *Eur J Cancer* 1999, **35**, 609.
16. Airley R, Loncaster J, Davidson S, *et al*. Glucose transporter Glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. *Clin Cancer Res* 2001, **7**, 928–934.
17. Loncaster JA, Harris AL, Davidson SE, *et al*. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Res* 2001, **61**, 6394–6399.
18. Koukourakis MI, Giatromanolaki A, Sivridis E, *et al*. Hypoxia-regulated carbonic anhydrase-9 (CA9) relates to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy. *Clin Cancer Res* 2001, **7**, 3399–3403.
19. Baer S, Casaubon L, Schwartz MR, Marcogliese A, Younes M. Glut3 expression in biopsy specimens of laryngeal carcinoma is associated with poor survival. *Laryngoscope* 2002, **112**, 393–396.
20. Reisser C, Eichhorn K, Herold-Mende C, Born AI, Bannasch P. Expression of facilitative glucose transport proteins during development of squamous cell carcinomas of the head and neck. *Int J Cancer* 1999, **80**, 194–198.
21. Kunkel M, Reichert TE, Benz P, *et al*. Overexpression of Glut-1 and increased glucose metabolism in tumors are associated with a poor prognosis in patients with oral squamous cell carcinoma. *Cancer* 2003, **97**, 1015–1024.
22. Kato H, Takita J, Miyazaki T, *et al*. Glut-1 glucose transporter expression in esophageal squamous cell carcinoma is associated with tumor aggressiveness. *Anticancer Res* 2002, **22**, 2635–2639.
23. Brizel DM, Schroeder T, Scher RL, *et al*. Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. *Int J Radiat Oncol Biol Phys* 2001, **51**, 349–353.
24. Bernier J, Denekamp J, Rojas A, *et al*. ARCON: accelerated radiotherapy with carbogen and nicotinamide in head and neck squamous cell carcinomas. The experience of the Cooperative Group of Radiotherapy of the European Organization for Research and Treatment of Cancer (EORTC). *Radiother Oncol* 2000, **55**, 111–119.
25. Kaanders JHAM, Bussink J, van der Kogel AJ. ARCON: a novel biology-based approach in radiotherapy. *Lancet Oncol* 2002, **3**, 728–737.
26. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 1996, **56**, 4509–4515.
27. Noguchi Y, Saito A, Miyagi Y, *et al*. Suppression of facilitative glucose transporter 1 mRNA can suppress tumor growth. *Cancer Lett* 2000, **154**, 175–182.