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In vivo imaging of cellular proliferation in colorectal cancer using positron emission tomodraphy

D L Francis, A Freeman, D Visvikis, D C Costa, S K Luthra, M Novelli, I Taylor, P J Ell

Background and aims: Positron emission tomodraphy (PET) using 18F labelled 2-fluoro-2-deoxy-D-glucose (18FDG) is an established imaging tool, although the recent development of a biologically stable thymidine analogue [18F] 3'-deoxy-3-fluorothymidine (18FLT) has allowed PET to image cellular proliferation by utilising the salvage pathway of DNA synthesis. In this study, we have compared uptake of 18FLT and 18FDG with MIB-1 immunohistochemistry to evaluate the role of PET in quantifying in vivo cellular proliferation in colorectal cancer (CRC).

Patients and methods: Patients with resectable, primary, or recurrent CRC were prospectively studied. Thirteen lesions from 10 patients (five males, five females), median age 68 years (range 54–87), were evaluated. Patients underwent 18FDG and 18FLT PET scanning. Tracer uptake within lesions was quantified using standardised uptake values (SUVs). Histopathological examination and MIB-1 immunohistochemistry were performed on all lesions, and proliferation quantified by calculating a labelling index (% of MIB-1 positively stained nuclei within 1500 tumour cells).

Results: Histology confirmed adenocarcinoma in 12 of 13 lesions; the remaining lesion was reactive. All eight intrahepatic lesions were visualised using both 18FDG and 18FLT. Three of the five resected liver metastases were also avid for 18FLT and showed high proliferation, while the remaining two lesions which demonstrated no uptake of 18FLT had correspondingly very low proliferation. There was a statistically significant positive correlation (r = 0.8, p < 0.01) between SUVs of the tumours visualised with 18FLT and the corresponding MIB-1 labelling indices. No such correlation was demonstrated with 18FDG avid lesions (r = 0.4).

Conclusions: 18FLT PET correlates with cellular proliferation markers in both primary and metastatic CRC. This technique could provide a mechanism for in vivo grading of malignancy and early prediction of response to adjuvant chemotherapy.
and low or absent in quiescent cells. High TK levels have been shown to be an important risk factor in patients with breast cancer and in addition in vitro studies have demonstrated a positive correlation between FLT uptake, cell growth, and TK activity.

The aim of this prospective study was thus to compare tumour uptake by both 18FLT and 18FDG PET and investigate their correlation with cellular proliferation using MIB-1 immunohistochemistry. To our knowledge this is the first study evaluating the role of PET in quantifying in vivo cellular proliferation in CRC.

PATIENTS AND METHODS

Patient selection
Ten patients (five males, five females), median age 68 years (range 54–87), with 13 resectable primary or recurrent colorectal cancers, were recruited at diagnosis from local surgical clinics. Demographics are given in table 1. Written informed consent was obtained following approval from the Local Research Ethics Committee and the Administration of Radioactive Substances Advisory Committee. All patients underwent whole body 18FDG scanning followed by an 18FLT scan (median intervening period 5 days (range 1–19)).

Imaging
18FDG was prepared using a modification of the technique described by Hamacher and colleagues27 while 18FLT was prepared according to the method of Cleij and colleagues.26 Radiochemical purity was >99% and >95% for 18FDG and 18FLT, respectively. All patients were asked to fast for six hours prior to scanning, and blood glucose levels were checked using a glucometer (Glucometer Elite; Bayer Pharmaceuticals, Newbury, Berkshire, UK) prior to injection of the tracers. All patients were normoglycaemic with a mean blood glucose 2 hours prior to scanning, and blood glucose levels were checked using a glucometer (Glucometer Elite; Bayer Pharmaceuticals, Newbury, Berkshire, UK) prior to injection of the tracers. All patients were normoglycaemic with a mean blood glucose

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The average SUV of five consecutive slices was obtained. The slices selected comprised the one with the maximum count density over each ROI and the four immediately adjacent slices.

Immunohistochemistry
Routine histological examination was performed on 4 μm thick haematoxylin and eosin (H&E) stained sections. Each specimen was sampled in a routine manner for diagnostic purposes in accordance with established Royal College of Pathology clinical guidelines. Immunohistochemistry for Ki-67 using the MIB-1 antibody was then performed on routine diagnostic blocks using 3 μm thick sections cut from paraffin blocks and dried overnight at 60°C. Sections were taken from xylene (two changes), through graded alcohols (100% and 70%) to water. Antigen retrieval was performed by pressure cooking for two minutes in a conventional 15 lb pressure cooker. Slides were then rinsed in 0.05% Tween 20 in Tris buffered saline (TBS/Tween). Endogenous peroxidase activity was blocked for 10 minutes using a commercially available peroxidase blocking solution (Dako, Ely, Cambridgeshire, UK). Primary mouse monoclonal antibody (MIB-1 (Dako) diluted 1/50 in TBS, pH 7.4) was applied for 60 minutes at room temperature.

Sections were rinsed in TBS/Tween and secondary antibody (goat anti-rabbit/mouse; Dako) was applied for 30 minutes. Sections were then rinsed in TBS/Tween and streptavidin-horseradish peroxidase (Dako) was applied for 30 minutes. Sections were again rinsed in TBS/Tween and diaminobenzidine was applied. Sections were dehydrated through graded alcohols (70% and 100%), cleared in xylene (two changes), and coverslipped with DPX mountant.

Examination of sections
Sections of tumour were identified on the H&E slides and the corresponding MIB-1 sections were examined independently.

Table 1 Patient characteristics, including calculated standardised uptake values and labelling indices for the respective lesions

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Weight (kg)</th>
<th>Resected lesions</th>
<th>18FDG (SUV)</th>
<th>18FLT (SUV)</th>
<th>LI (% MIB-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>87</td>
<td>72</td>
<td>Primary (asc colon)</td>
<td>9.2</td>
<td>3.5</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>66</td>
<td>82</td>
<td>Peritoneal recurrence</td>
<td>7.6</td>
<td>3.3</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>78</td>
<td>54</td>
<td>Peritoneal lesion</td>
<td>7.8</td>
<td>—</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>75</td>
<td>63</td>
<td>Liver metastases 1</td>
<td>5.5</td>
<td>—</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>62</td>
<td>85</td>
<td>Liver metastases 2*</td>
<td>11</td>
<td>—</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>60</td>
<td>58</td>
<td>Primary (rectal)</td>
<td>9.6</td>
<td>4.8</td>
<td>70</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>59</td>
<td>70</td>
<td>Liver metastases</td>
<td>3.7</td>
<td>9.1</td>
<td>87</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>54</td>
<td>55</td>
<td>Primary (caecal)</td>
<td>9.7</td>
<td>5.5</td>
<td>77</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>67</td>
<td>95</td>
<td>Liver metastases</td>
<td>3.7</td>
<td>4.8</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>71</td>
<td>65</td>
<td>Primary (trans colon)</td>
<td>8.5</td>
<td>2.9</td>
<td>67</td>
</tr>
</tbody>
</table>

18FDG, 18F labelled 2-fluoro-2-deoxy-D-glucose; 18FLT, 3’-deoxy-3-fluorothymidine; SUVs, standardised uptake values; LI, labelling index.

*Those lesions seen as avid for 18FDG but not for 18FLT
†Inflammatory lesions not demonstrating uptake for 18FLT.
Statistical analysis

Interobserver reliability was assessed using Pearson’s correlation coefficient, and linear regression analysis using the SPSS statistical package was employed to correlate uptake of both 18F FLT and 18FDG with MIB-1.

RESULTS

Histopathology of all lesions (n = 13, five primary, five liver metastases, and three peritoneal) confirmed adenocarcinoma in 12 of 13 cases. The remaining case was thought to be a malignant peritoneal recurrence by preoperative CT, 18FDG PET imaging, and at intraoperative evaluation but on microscopic histological examination was found to be an inflammatory lesion secondary to fat necrosis.

There was excellent interrater reliability between the two observers responsible for quantifying proliferation using MIB-1 immunohistochemistry (Pearson’s correlation coefficient 0.9) (fig 1).

Quantification of tracer uptake in addition to the corresponding LIs can be seen in table 1. All five primary tumours in addition to both malignant peritoneal lesions were visualised with increased focal uptake using both 18F FLT and 18FDG. Three of five liver metastases were clearly seen to be avid for 18F FLT, and all showed a corresponding high proliferation LI.

The three lesions (peritoneal inflammatory lesion and two liver metastases) which did not show increased uptake of 18F FLT and were therefore not visualised using this tracer had corresponding low proliferation rates, as determined by the LIs, ranging from 19 to 26.

SUVs for those lesions seen with 18FDG ranged between 3.7 and 11 compared with a range of 1.4–9.1 for the 10 lesions visualised using 18F FLT.

There was a statistically significant correlation (r = 0.8, p<0.01) between SUVs of the 10 tumours visualised with 18F FLT and the corresponding LIs (fig 2). No such correlation

by two experienced observers, A and B, blinded to the results of the scans.

Three MIB-1 stained sections of tumour were used for each lesion to account for tumour heterogeneity. Thirty high power fields (>40 objective lens) were examined and 50 tumour cells were assessed per field using a graticule, making a total of 1500 cells per case. A labelling index (LI) was calculated as the percentage of positive staining nuclei out of all nuclei examined. “Positive” nuclear staining was regarded as staining of the entire nucleus. Nuclei with no staining or nucleolar staining only was regarded as negative.

Figure 2 Linear regression analysis for those lesions seen with 18F labelled 3-deoxy-3-fluorothymidine (18FLT) (n = 10), correlating labelling indices (LIs) with standardised uptake values (SUVs) (r = 0.8).

Figure 3 Linear regression analysis for those lesions seen with 18F labelled 2-fluoro-2-deoxy-D-glucose (18FDG) (n = 13), correlating labelling indices (LIs) with standardised uptake values (SUVs) (r = 0.4).

(r = 0.4) was demonstrated in the case of 18FDG avid lesions (fig 3).

Differences in tracer uptake compared with macroscopic histology and MIB-1 immunohistochemistry are highlighted in fig 4.

DISCUSSION

Our results suggest that 18F FLT reflects proliferative activity in CRC, as determined by MIB-1 staining. Lack of correlation between LIs and 18FDG supports previous evidence that 18FDG is not a specific marker of proliferation. The lesions successfully imaged with 18FLT demonstrated high SUVs in addition to high LIs. On the other hand, those lesions not visualised by 18F FLT were shown to have correspondingly low LIs.

It is known that glucuronidation of 18F FLT within the liver leads to high hepatic background activity which may inhibit its diagnostic utility. This was exemplified by failure of 18F FLT PET to show two of the five liver metastases against this high liver background. However, both of these lesions displayed low proliferation labelling indices (19% and 26%), suggesting relatively slow growth compared with the remaining three liver metastases (labelling indices of 87%, 87%, and 73%). The fact that certain liver lesions are not seen with 18F FLT may have prognostic implications in terms of the aggressiveness of these tumours.

The well documented problems of specificity due to uptake of 18FDG by activated macrophages is also highlighted in this study by the peritoneal lesion thought to be malignant because of a high uptake of 18FDG. Microscopic examination of the entire lesion showed a florid inflammatory response and fat necrosis secondary to previous surgery, but no tumour. In contrast, 18F FLT showed no increased uptake within this lesion, suggesting that this new tracer may have a role to play in improving the diagnostic specificity of PET.
18FLT PET has recently been found in the investigation of solitary pulmonary nodules to be specific for malignant lesions. In addition, 18FLT correlates with the Ki-67 labelling index and estimation of prognosis. This hypothesis has been further validated by Vesselle and colleagues who assessed proliferation rates in 11 histologically proven non-small cell lung cancers using 18FLT PET and again demonstrated significant correlation with Ki-67.

The very nature of PET allows metabolic assessment of the whole tumour in its heterogeneic entirety. This coupled with the unique ability to quantitate the uptake of the tracers used, non-invasively and in vivo, has opened new avenues in the assessment of malignancy by functional imaging.

A system for grading the aggressiveness of CRC was first developed by Cuthbert Duke in 1932, and it was not until 1995 that a classification of prognostic markers was first suggested by the College of American Pathologists. Seventy years on, Dukes and TNM, along with the histological grade of the tumour, still remain the only reliable methods to dictate whether patients with CRC will receive adjuvant therapy. Unfortunately, under current guidelines, which have been set up in light of these prognostic indicators, there is still a cohort of patients who unnecessarily receive toxic chemotherapy while others who could potentially benefit from it are not recommended to receive it. In view of this, the prognostic value of Ki-67 in colorectal cancer has recently been revisited by the National Surgical Adjuvant Breast and Bowel Project Collaborative Study group in an attempt to define a marker on which therapeutic decisions could be made with greater precision.

Ki-67 was selected for investigation because of its potential value in identifying populations of individuals who may or may not benefit from adjuvant chemotherapy. Significant prognostic value was found for patients with Dukes' B and C disease although it was unable to differentiate between groups who might benefit from adjuvant chemotherapy. Prior to this study, there have been contradictory reports regarding the role of Ki-67 as a prognostic indicator in CRC. Palmqvist and colleagues showed that those tumours with low Ki-67 expression at the invasive margin had a poor prognosis while Kimura and colleagues concluded that the tumours with high expression at the site of deepest invasion had a worse prognosis. In contrast, both Kubota and colleagues who analysed 100 cases of CRC using Ki-67, and Kyzer and colleagues who examined 30 resected specimens of CRC, concluded that Ki-67 could not be used as a prognostic determinant in CRC.

Our study has demonstrated that 18FLT PET may be used as a non-invasive method of quantitating cellular proliferation in CRC. This may offer the opportunity for CRC to be imaged at a pretreatment stage for those undergoing surgery and perhaps define the population of patients who might benefit from neoadjuvant therapy. In addition, it may also help in identifying a group of patients with recurrent disease who would not benefit from firstline chemotherapy as a result of the low proliferation rate of the tumour, as demonstrated by the low SUV on 18FLT PET imaging.

This would offer the clinician a further prognostic marker with which to assess the aggressiveness of the disease and perhaps allow a more precise method for therapeutic decisions to be made. In real terms, a decision regarding response to chemotherapy in CRC is usually judged six weeks into a 12 week treatment cycle. The potential of avoiding six weeks of unnecessary toxic chemotherapy by differentiating the non-responders from the responders at a pretreatment stage is a prospect which cannot be ignored.

Future work will help in validating 18FLT PET as a potential tool for the non-invasive in vivo assessment of CRC and may

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**Figure 4.** Primary tumour of the caecum (A) and two liver metastases (B, C) with their respective positron emission tomography images, with both 18F labelled 2-fluoro-2-deoxy-D-glucose (18FDG) and 18F labelled 3-deoxy-3-fluorothymidine (18FLT). Corresponding MIB-1 slides are shown at x40 power, with positive nuclei staining brown. The liver lesion (B) which stains very highly for MIB-1 (LI 77%) is seen as avid for 18FLT in comparison with the liver lesion (C) with a low MIB-1 stain (LI 19%) which is not seen with 18FLT. The photopenic appearance of the liver lesion (C) highlights the increased hepatic background activity secondary to glucuronidation of 18FLT in contrast with the low uptake in the lesion by virtue of its low proliferative rate.

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18FDG PET imaging involves the use of 
18FDG, which is a glucose analogue that is transported across the cell membrane and phosphorylated by hexokinase to 
18F6D-glucose 1-phosphate and 18FDG-6-phosphate. The 18FDG-6-phosphate is further processed by the glycolytic pathway to 
18FDG-1,6-diphosphate, which is trapped in the cell due to the irreversible reaction catalysed by phosphoglucomutase. The accumulation of 18FDG in the cell can then be detected by PET scanning. The uptake of 18FDG is directly related to glucose metabolism, which is a hallmark of cell proliferation. Therefore, 18FDG PET imaging can be used to assess the metabolic activity of tumours and to evaluate the response to therapy. However, 18FDG PET imaging has limitations, such as the difficulty in distinguishing between necrosis and residual tumour, and the lack of specificity in identifying the presence of recurrent disease. In contrast, 18FLT PET imaging is based on the principle that tumour cells have increased rates of DNA synthesis, which is reflected in increased 3-deoxy-3-fluorothymidine incorporation into DNA. The uptake of 18FLT is directly related to the rate of DNA synthesis, which is a marker of cell proliferation. Therefore, 18FLT PET imaging can be used to assess the proliferative activity of tumours and to evaluate the response to therapy. However, 18FLT PET imaging also has limitations, such as the difficulty in distinguishing between active and non-active lesions, and the lack of specificity in identifying the presence of recurrent disease.
enable more accurate evaluation of the antitumoral effects of new as well as established anticancer drug treatments aimed at competing with DNA synthesis.

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