Inverse relationship between the expression of the human papillomavirus type 16 transcription factor E2 and virus DNA copy number during the progression of cervical intraepithelial neoplasia

Mark Stevenson,1 Lucy C. Hudson,1 Julie E. Burns,1 Roy L. Stewart,2 Michael Wells2 and Norman J. Maitland1

1 YCR Cancer Research Unit, Department of Biology, University of York, York YO10 5YW, UK
2 Division of Oncology and Cellular Pathology, University of Sheffield Medical School, Sheffield S10 2RX, UK

The human papillomavirus type 16 (HPV-16) status of 43 cervical biopsies, which had been characterized histologically as normal, various grades of cervical intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma, was examined by using (i) a novel antibody against the HPV-16 E2 protein, (ii) sensitive HPV-16 DNA in situ hybridization and (iii) microdissection/PCR for the E2 ORF. The data indicate that E2 protein expression is highest in koilocytes in lower-grade CIN (I), but decreases with increasing grade, whereas the detection of HPV DNA is delayed until CIN I/II, rising to the highest levels in carcinoma cells. Co-localization of E2 with HPV-16 DNA-positive cells was most commonly observed in koilocytes in CIN II lesions. PCR analyses of microdissected epithelium from the same or serial sections indicated that E2 ORFs were retained in an intact form in a number of higher-grade CIN lesions and invasive carcinomas.

There are in excess of 80 human papillomavirus (HPV) types, of which over 20 are able to infect the male or female anogenital tract (HPV database; http://hpv-web.lanl.gov/). While most HPV-associated lesions will remain benign or will regress spontaneously, approximately 15% of all HPV-positive cases will progress to high-grade cervical intraepithelial neoplasia (CIN) (Fowler, 1993; DiSaia & Creasman, 1989) and cancers, which almost invariably contain DNA of high-risk HPV types, e.g. HPV-16 (zur Hausen, 1994; Richart et al., 1998). Viral genomes are maintained at low copy numbers as extrachromosomal episomes in the undifferentiated basal stem cells and in higher copy numbers in the more differentiated superficial cells, as detected by in situ hybridization (ISH). Since detection limits are normally higher than 20 HPV genome copies per cell, positive ISH is often an indicator of HPV genome replication and is most frequently associated with koilocytes within CIN I and II lesions (Schneider et al., 1987; Resnick et al., 1996; Ziol et al., 1998).

The HPV E2 transcription factor plays a critical role in both the activation and repression of virus transcription (Bouvard et al., 1994) and viral DNA replication (Chow & Broker, 1994). It consists of a C-terminal DNA binding/dimerization domain, linked by a flexible hinge region to an N-terminal transactivation domain (Ham et al., 1991; Sanders et al., 1995). Maitland et al. (1998) used polyclonal antibodies, specific for the C-terminal region of the E2 protein of HPV-16 (Sanders et al., 1995), to determine the expression patterns of HPV-16 E2 in a series of single cervical tissue sections presenting low- and high-grade CIN and invasive carcinomas. Nuclear staining was localized to both superficial and basal layers of the cervical epithelium and was highest in koilocytic lesions within CIN I, but was lower or absent in higher-grade CIN lesions and carcinomas.

In order to determine the relationship between the expression patterns of HPV-16 E2 and the centres of HPV DNA replication as revealed by ISH, a panel of 43 formalin-fixed, paraaffin-embedded cervical biopsy specimens was obtained from the surgical pathology archives of the Division of Oncology and Cellular Pathology, University of Sheffield Medical School, Sheffield, UK. Twenty-five biopsies were chosen for the diagnosis of CIN grade I, II or III and eight for invasive squamous cell carcinoma (SCC). The remaining 10 specimens were histologically normal. Several individual 5 µm sections were cut from each formalin-fixed block and mounted on 3-aminopropyltriethoxysilane-coated slides. Sections of human tonsil were cut for use as negative controls.
HPV-16 DNA was detected by ISH with an FITC-labelled HPV-16 probe (BioGenex), as described by the manufacturer. Typical results are shown in Fig. 1(A) and are summarized in Table 1. Following ISH, a strong signal covering the entire nucleus was observed in nine sections (all high-grade CIN and SCC), with a further nine sections having weaker punctate nuclear staining. In CIN, the strongest staining was observed in the koilocytes [see Fig. 1A(c)], which are morphologically characteristic of HPV infection and probably indicate sites of viral DNA replication in the superficial layers of the epithelium.
Invasive SCC exhibited homogeneous nuclear staining in foci of infected cells throughout the sections. The ISH signal intensity increased from punctate nuclear staining in the parabasal and intermediate layers [Fig. 1A(g), inset] to homogeneous, strong nuclear staining in the superficial layers, confirming an increase in virus copy number, as has been reported previously (Schneider et al., 1987; Resnick et al., 1996; Ziol et al., 1998).

Serial sections to those used above were incubated with a polyclonal anti-E2 antiserum with reactivity against the entire E2 molecule, prepared as described by Sanders et al. (1995). The antiserum detected a single species in Western blotting and labelled insect cells infected with E2 expressing recombinant baculoviruses (data not shown). Immunohistochemistry (IHC) was performed by using the streptavidin–biotin–peroxidase complex technique (Maitland et al., 1998). As illustrated in Fig. 1(A), high levels of E2 protein expression were detected in seven sections, whilst low-level expression was detected in six sections (Table 1). In contrast to the ISH results, the strongest immunoreactivity was generally observed in areas of CIN grades I and II. Weaker immunoreactivity was present in CIN grade III and no E2 was detectable in SCC, although two invasive sections had associated areas of lower-grade CIN that were immunoreactive. In the CIN lesions, expression was confined to the superficial layers of the epithelium and specifically to the koilocytic nuclei, with little immunoreactivity of the intermediate or basal layers. E2 expression was localized to both nuclear and cytoplasmic regions of superficial cells, in contrast to our previous results with the antiserum against the C terminus of E2, where the immunoreactivity was predominantly nuclear (Maitland et al., 1998).

PCR analysis of microdissected areas from tissue sections screened previously was carried out as described by Macintosh et al. (1998) with overlapping HPV-16 E2-specific primers (see Fig. 2A). Of the 43 samples, 38 gave a PCR product of the expected size (267 bp) with a cellular gene control, hypoxanthine phosphoribosyl transferase (HPRT), and were then analysed with four primer pairs covering the E2 ORF (Fig. 2B and Table 1). DNA extracted from CaSki cells (containing 500–600 copies of the HPV-16 genome) or SiHa (one copy of HPV-16; not shown) was used as positive controls. Primer sensitivity varied only 2-fold between the primers that detected the N and C termini of the E2 ORF and was reproducibly equivalent to less than one copy of HPV-16 DNA per cell without further enhancement.

The PCR data are summarized in Table 1 and indicate a good correlation between the presence of HPV DNA (by both PCR and ISH) and positive E2 staining with the polyclonal antibody. Significantly, all 10 normal cervical samples failed to generate a PCR product with any of the E2 primer pairs and of four samples exhibiting CIN I, only one produced positive results with all four E2-specific primer pairs. This was taken as confirmation that the E2 ORF was intact in this lesion. With increasing CIN grade, the situation became more complex, even after microdissection. Lack of detection of internal E2

![Fig. 1. (A) Cervical epithelial tissue sections following ISH with an HPV-16 DNA probe and IHC with a novel anti-E2 antiserum. Normal cervical epithelium revealed no detectable viral DNA following ISH (a) or E2 protein expression following IHC (b) (the red border surrounding the epithelial layers and including the basement membrane illustrates the extent of tissue removed by microdissection for subsequent PCR analysis). CIN II tissue subjected to ISH revealed positively stained koilocytic nuclei located in the superficial layers of the epithelium (c), indicating centres of viral DNA replication. CIN II tissue subjected to IHC (d) showed positive nuclear and cytoplasmic immunoreactivity within the superficial layers of the epithelium, revealing E2 protein expression. A correlation was observed between the epithelial regions that contained viral DNA and that stained positively for E2 protein expression in CIN II (e and f) and CIN III (g and h). Following ISH, both homogenous nuclear staining (e.g., g; up arrows) and punctate signals (e, down arrow; g, down arrow and inset) were detected. Localized nuclear E2 expression is indicated by an arrow (h). Section (a) was counterstained with Neutral red, whilst sections (b) and (f) were counterstained with haematoxylin and bleud with tap water. Sections (c), (d), (e), (g) and (h) were not counterstained. (B) Summary of the proportion of samples that were HPV-16 positive following ISH (shaded bars), IHC (hatched bars) or both ISH and IHC (filled bars) in normal cervix, various grades of CIN and SCC. *, E2 expression was located in an epithelial region of pre-invasive neoplasia and not in areas of invasive SCC.](https://www.microbiologyresearch.org/1827)
Fig. 2. For legend see facing page.
fragments was taken as evidence of E2 ORF rearrangement or deletion in 2/7 CIN II and 1/10 CIN III tissues. However, of the eight DNA samples extracted from areas of invasive SCC, four were positive with all of the E2 primers and a further three with the C-terminal primers only.

The combined IHC, ISH and PCR results can be summarized as follows. (i) HPV-16 was undetectable in 9/10 (90%) of the normal biopsies, whereas 25/33 (76%) CIN/cancer samples were positive for HPV-16 detection by at least one detection method. (ii) There was good agreement between the PCR-determined status of the E2 gene and the detection of viral DNA by ISH. A positive result was generated from both C- and N-terminal primers in normal sections (lanes 1–10) and sections with invasive carcinoma (lanes 41–48), respectively. Gels (g)–(h) illustrate the levels of HPV-16 DNA in normal (lanes 1–10) and invasive samples (lanes 41–48) by using primers that are specific to the E2 hinge N-terminal and C-terminal ends, respectively. (C) Summary of the relative proportion of samples that were HPV-16 positive after PCR analysis with each of the four primer sets directed towards the E2 N terminus (open bars), the N-terminal (shaded bars) and C-terminal (hatched bars) regions of the hinge and the C terminus (filled bars).

![Fig. 2](image)

**Fig. 2.** (A) HPV-16 E2 ORF, (B) HPV-16 E2 ORF N-terminal, (C) HPV-16 E2 ORF C-terminal. HPV-16 E2 ORF N-terminal (open bars), N-terminal (shaded bars) and C-terminal (hatched bars) regions of the hinge and the C terminus (filled bars).

HPVs, since the anti-HPV-16 E2 antisera does not cross-react with low-risk HPV E2.

When the trend in detection with increasing severity of lesions is plotted, as shown in Fig. 1 (B), the frequency of HPV DNA detection rose in the koilocytic cells while the frequency of E2 protein detection declined, although the E2 ORF was still present. Thus, despite the higher frequency of HPV-16 DNA detection due to the amplification of HPV genomes with increasing severity of lesion, E2 protein expression is apparently downregulated in CIN grade III and carcinoma in situ.

In experimental systems, transfection of the HPV-16 E2 ORF has been shown to induce apoptosis in both HPV-transformed and non-transformed cell lines either via either a p53-dependent (Webster et al., 2000) or -independent (Desaintes et al., 1999) pathway. Thus, it is possible that squamous epithelial cells that express high levels of E2 in early CIN lesions may subsequently undergo apoptosis. Significantly, overexpression of E2 has not been detected in human tissue cultures by IHC, although the biological activity can be detected by gel-shift analysis of E2-binding site DNA and by transactivation assays (C. Schmitz, personal communication; our unpublished data). The reduction in E2 expression with increasing grade of CIN is supported by the change in anti-E2 IgA from high levels in low-grade CIN to low or undetectable levels in high-grade CIN (Rocha-Zavaleta et al., 1997) and by
Table 1. Summary of HPV-16 detection by ISH, IHC and PCR analysis

Observations are scored as: +, strong positive; ±, weak positive; —, negative; ND, not determined.
Abbreviations: Norm, normal tissue sample; Inv, invasive carcinoma; term., terminus.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>Norm</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>CIN I</td>
<td>—</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>13</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>15</td>
<td>CIN I</td>
<td>—</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>17</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>18</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>19</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>20</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>21</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>22</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>23</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>24</td>
<td>CIN I</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>25</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>26</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>27</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>28</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>29</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>30</td>
<td>CIN I</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>31</td>
<td>CIN II</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>32</td>
<td>CIN II</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>33</td>
<td>CIN II</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>34</td>
<td>CIN II</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>35</td>
<td>CIN II</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>36</td>
<td>CIN II</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>37</td>
<td>CIN II</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>38</td>
<td>CIN II</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>39</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>40</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>41</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>42</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>43</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>44</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>45</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>46</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>47</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>48</td>
<td>CIN III</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*E2 protein expression was located in a region of pre-invasive neoplasia and not areas of invasive SCC. Note that there are no samples 11, 16, 20, 21 or 26.

Previous attempts to relate HPV DNA replication to patterns of HPV E2 gene expression have been restricted by
the poor sensitivity of antibodies to E2 and the difficulties posed by overlapping reading frames and complex HPV
transcription patterns (for E2 and E4) in the design of probes for RNA detection (Higgins et al., 1991). The dual role of the
E2 protein in transcriptional activation/repression (Bouvard et al., 1994) and in viral DNA replication, both in transient systems (Chow & Broker, 1994) and in HPV-31-infected keratinocyte raft cultures (Stubenrauch et al., 1998), suggests that co-localization of E2 and high levels of HPV DNA is to be expected.

Parallels can be drawn with the simian virus 40 (SV-40) system (reviewed by Singer & Berg, 1991), in which the T antigen displays many of the activities separated into E1 and E2 in HPV. During a productive infection with SV-40, the high early levels of T antigen protein acting as a transactivator (E2-like) are suppressed increasingly by self-regulation of transcription from the early promoter as T antigen binding to the origin and DNA replication (requiring T antigen in its E1-like role) begin to dominate. Since the half-life of HPV-16 E2 in human cells is extremely short (C. Schmitz, personal communication) and its main role in DNA replication is as an initiator of DNA replication (Del Vecchio et al., 1992; Chiang et al., 1992), by locating the E1 protein to the origin of replication (Sedman & Stenlund, 1996; Lusky et al., 1994), a fall in E2 levels below our detection limits is not unexpected as HPV DNA levels increase.

Progression from low-grade to high-grade neoplasia is often accompanied by integration of the virus genome into the host chromosome, disrupting or deleting the E2 ORF (Schwarz et al., 1985; Yee et al., 1985; Krajinovic et al., 1993; Kalantari et al., 1998) with inactivation of the E2 regulatory protein. However, many tumours have also been reported to contain multiple copies of circular, episomal, non-disrupted viral genomes (Matsukura et al., 1989). Our PCR analysis confirms the existence of the intact E2 ORF in seven pre-invasive and four invasive lesions (all in ISH-positive cells). While the ISH signals could be the result of tandem copies of HPV integrated within the chromosome (as in CaSki cells), the diffuse pattern of the positive signal in most cases (e.g. Fig. 1A(a)) and the change from a punctate to a pan-nuclear signal towards the more differentiated superficial layers of epithelium is more indicative of pan-nuclear episomally replicating HPV copies.

Our results indicate that detectable E2 protein levels can reveal the presence of a high-risk HPV infection, certainly in advance of the ability to detect multiple copies of HPV DNA in CIN lesions. This could therefore provide an earlier marker for at-risk patients and potential neoplastic progression in HPV-infected cells as part of a cervical screening programme.

We would like to thank Nicky Rolley, Jacky Knight and Kath Ramsey for technical support, Jo Birch for reference organization and Meg Stark for photographic preparation. This work was funded by Yorkshire Cancer Research.

References


M. Stevenson and others


Received 23 December 1999; Accepted 8 March 2000