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ORIGINAL ARTICLE

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# Heterogeneity of expression of immunohistochemical tumour markers in testicular carcinoma in situ: pathogenetic relevance

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**Abstract** Testicular carcinoma in situ (CIS) is the precursor of germ cell tumours in adults, except for spermatocytic seminoma. The mechanism of the progression from premalignant CIS to invasive and overt tumours is largely unknown. There are currently two main hypotheses: one is that CIS can progress directly to either seminoma or nonseminoma; according to the other, seminoma is the intermediate stage between CIS and nonseminoma. CIS cells express several tumour antigens, such as placental-like alkaline phosphatase (PLAP), TRA-1–60, or the c-*kit* proto-oncogene protein product (Kit), which are present to varying degrees in the invasive germ cell tumours. In this study, CIS cells adjacent to either pure or combined tumours were examined by double immunohistochemical staining for simultaneous expression of TRA-1–60 (typical for embryonal carcinoma) and either Kit (expressed by seminomas) or PLAP (found mainly in seminomas, but also in some cases of nonseminoma). Marked differences in the expression of these antigens were found among CIS cells, especially those adjacent to mixed tumours. We conclude that CIS is a phenotypically heterogeneous lesion, and that the CIS cells, despite identical morphology and close spatial localization, may be in different stages of progression. The results lend support to the hypothesis that CIS can progress directly to both seminomatous and nonseminomatous tumours.

**Key words** Testicular neoplasms · Testicular carcinoma in situ · Tumour markers · c-*kit* · Placental-like alkaline phosphatase · TRA-1–60 · Double immunohistochemical staining

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## Introduction

Testicular carcinoma in situ (CIS) [35], also known as intratubular germ cell neoplasia (ITGCN) [2], gonocytoma in situ [14], or testicular intraepithelial neoplasia (TIN) [22], is generally recognized as the precursor of overt germ cell tumours in adults, except for spermatocytic seminoma [37].

Despite a common origin from CIS, germ cell tumours comprise a wide variety of histological forms. According to the classification of Pugh [32], there are two main subtypes, seminoma (including classic and spermatocytic seminoma) and teratoma, which includes all nonseminomatous germ cell tumours (embryonal carcinoma, malignant and differentiated teratoma, trophoblastic teratoma and yolk sac tumour). There are also combined, or mixed, tumours, which contain both classic seminoma and various nonseminomatous components. Spermatocytic seminoma is not associated with CIS and arises in older men, and this is now widely considered as a separate histogenetic entity [6, 26]. For simplicity, three clinicopathological categories are used in this paper: seminoma (classic only), nonseminoma, and combined tumours.

The pathogenetic relationship between CIS and various types of overt tumours is still unknown [4]. There are two main hypothetical models. One of them suggests that seminoma is the intermediate stage between a precursor germ cell and various forms of tumours included in a general group of nonseminomas. This model was set up mainly on the basis of the striking phenotypic resemblance of seminoma to the precursor CIS cell, on the relative DNA values, which in most reports were higher in CIS and seminomas (nearly tetraploid) than in nonseminomas (low triploid) [12, 25, 27, 29], and on the results of comparative cytogenetic studies [17] and pathomorphological observations [3, 28, 33, 38, 41]. The other model of tumour progression suggests that seminoma and nonseminoma arise independently from a precursor germ cell. This model has been supported by several authors, including our group [5, 31, 36]. We believe that

the precursor cell is, in fact, the CIS germ cell. The most recent studies, which are presented briefly in the discussion [8, 13, 21, 30], contain elements that can either support or contradict both models, thus suggesting that perhaps the combination of independent and linear progression is closest to reality.

In this study, we decided to re-examine the expression of several immunohistological tumour markers in CIS cells, using a double staining technique that allows evaluation of two different antigens at the same time. The rationale was that comparison of the expression of the cell surface antigens in the preinvasive CIS cells found in the vicinity of different invasive tumours that express these antigens to varying degrees might provide information on their pathogenetic relationship. Moreover, we have noticed that some markers, especially TRA-1–60, are not always expressed by all CIS cells. Therefore, the simultaneous expression of markers typical for seminoma and for embryonal carcinoma was analysed in CIS cells adjacent to both types of tumours, including the combined tumours, as well as in isolated CIS lesions that had not yet progressed to invasive malignancy.

## Materials and methods

The series included 14 orchidectomy specimens containing CIS; 3 of them were found in testicular parenchyma adjacent to the combined tumours (containing components of classic seminoma and various elements of nonseminoma, which are listed in Table 1); 3 CIS samples were associated with pure classic seminomas; 1 CIS sample, with an anaplastic seminoma; 3 were adjacent to nonseminomas (two of them were pure embryonal carcinomas); and finally, 4 specimens contained only CIS, without the presence of overt tumours. In those cases with isolated CIS, the lesion was found in testicular biopsies performed because of infertility, oligospermia or, in 1 case, an abnormal ultrasound pattern [11]. The specimens were divided into fragments, fixed in various fixatives (Stieve's, Bouin's and buffered formalin), dehydrated and embedded in paraffin. In this study, only specimens fixed in Stieve's or Bouin's were used, for good morphology and because the epitopes of the markers used in this study were preserved equally well.

The following antibodies, which are known to be good markers for CIS cells, were used: a rabbit polyclonal anti-placental-like alkaline phosphatase, PLAP (Cat. no. A0268, DAKO, Glostrup, Denmark), which is strongly expressed in seminoma and rather focally in embryonal carcinoma [9, 16, 19, 23]; a mouse monoclonal antibody TRA-1–60 (provided by Prof. P.W. Andrews, Sheffield, UK), which is very strongly expressed in embryonal carcinoma and to a variable degree but in most cases rather weakly expressed in seminoma [1, 10]; a rabbit polyclonal anti-c-*kit* protein product, Kit (Cat. no. SC-39, Santa Cruz Biotechnology, Santa Cruz, Calif.), which is expressed in seminoma but not in nonseminoma [34, 39]. The antibodies were used in the following combinations: (a) TRA-1–60 and c-*kit*; (b) TRA-1–60 and PLAP.

For immunohistochemistry, a double indirect immunostaining technique that uses a combination of a rabbit polyclonal and a mouse monoclonal antibody followed by peroxidase and alkaline phosphatase detection systems was applied. Briefly, tissue sections cut at 4 μm onto Vectabond-covered microscope slides were dewaxed, rehydrated and incubated for 30 min in 1% solution of $H_2O_2$ in methanol to quench the endogenous peroxidase activity. After rinsing in Tris buffer (pH=7.4), the sections were incubated with a mixture of two primary antibodies overnight at 4°C. For every sample, serial sections were also incubated with each of the antibodies separately and with a dilution buffer alone. All subsequent incubations and washes were performed at room temperature. After four washes in Tris buffer supplemented with 1% Triton X-100, the sections were incubated with a mixture of link antibodies (an alkaline phosphatase-conjugated porcine-anti-rabbit IgG from DAKO; and a biotinylated goat-anti-mouse IgG from Zymed, S. San Francisco, Calif.) for 45 min. After washing, the sections were incubated with the peroxidase-conjugated streptavidin (Zymed) for 30 min, then washed and equilibrated in Tris buff-

**Table 1** Expression of tumour markers in CIS cells adjacent to various types of germ cell tumours (histological type of the tumour is given in brackets: *CIS* carcinoma in situ, *SE* seminoma, *NS* nonseminoma, *EC* embryonal carcinoma, *ES* endodermal sinus tumour, *TE* teratoma, *comb.* combined tumour, ++ strong staining, + weak staining, – no staining detected)

| No. | Histology | Relative number of CIS cells positive for tumour markers | | |
|---|---|---|---|---|
| | | PLAP | Kit | TRA-1–60 |
| 1 | CIS only | 96% (++) | 87% (++) | (–)[a] |
| 2 | CIS only | 95% (++) | 76% (++) | 1% (++) |
| 3 | CIS only | 96% (++) | 52% (++) | (–) |
| 4 | CIS only | 82% (++) | 53% (++) | (–)[a] |
| 5 | CIS + SE | 97% (++)[b] | 84% (++)[b] | (–)[a, b] |
| 6 | CIS + SE | 97% (++) | 94% (++) | 2% (++) |
| 7 | CIS + SE | 95% (++) | 93% (++) | 2% (++) |
| 8 | CIS + SE[c] | 77% (++) | 18% (+) | 91% (++) |
| 9 | CIS + NS (EC) | 92% (++) | 73% (++) | 9% (++) |
| 10 | CIS + NS (EC) | 95% (++) | 72% (+) | 12% (++)[d] |
| 11 | CIS + NS (ES) | 94% (++) | 76% (++) | 7% (++)[d] |
| 12 | CIS + comb. (SE, EC, TE, ES) | 98% (++) | 73% (++) | 68% (++) |
| 13 | CIS + comb. (SE, EC, TE) | 97% (++) | 87% (++) | 13% (++)[e] |
| 14 | CIS + comb. (SE, EC-focally) | 91% (++) | 89% (++) | 10% (++) |

[a] Except 1–2 cells per section that express TRA exclusively (negative for PLAP and Kit)
[b] One CIS tubule found with 50% PLAP (+), Kit (–), 100% TRA (++)
[c] Anaplastic seminoma
[d] Cells positive for TRA grouped focally, most of them negative for other markers
[e] Most of TRA-expressing cells (in the cellular membrane) negative for other markers, but approx. 1/3 of cells positive for PLAP and Kit express intracellular TRA

**Table 2** Summary of the results showing the prevalent pattern of the expression of tumour markers in CIS cells (*CIS only* no overt tumour present, *CIS-SE* CIS adjacent to seminomas, *CIS-NS* CIS adjacent to nonseminomas; *CIS-SE+NS* CIS adjacent to combined tumours, – negative, + <2% positive, ++ 3–20% positive, +++ 20–75% positive, ++++ >75% positive)

| | TRA-1–60 | Kit | PLAP |
|---|---|---|---|
| CIS only | –/+ | +++/++++ | ++++ |
| CIS-SE[a] | –/+ | ++++ | ++++ |
| CIS-NS | +/++ (few tubuli ++++) | ++++ (few tubuli –) | ++++ (few tubuli –) |
| CIS-SE+NS | ++ (few tubuli ++++) | ++++ (few tubuli –) | +++/++++ (few tubuli –) |

[a] Case 8, an anaplastic seminoma was exluded from this summary

er, pH=8.7. For visualizing the activity of alkaline phosphatase, a solution of pH=8.0 of naphtol-AS-MX phosphate and either Fast Blue or New Fuchsin with 1 μM levamisole (all substrates from Sigma) was applied (incubation 20–30 min in the dark), producing a blue or a pink-violet reaction product, respectively. Enzymatic activity was stopped by washing in distilled water. Subsequently, peroxidase activity was visualized by applying a solution of aminoethylcarbazol (AEC; red product) or diaminobenzidine (DAB; dark brown product) with $H_2O_2$ for 15 min in the dark. Some of the slides were lightly counterstained by immersion in Mayer's haematoxylin for 3–5 s. After the final wash in distilled water, the sections were covered with glass coverslips using an aqueous mounting medium.

The sections were examined under a light microscope. An arbitrary score for the intensity of staining was used: (++): strongly positive; (+): weakly positive; (–): negative. Both positive and unstained CIS cells were counted and the results were expressed as the relative number (percentage) of positive CIS cells present in a section (Table 1). In Table 2 a semiquantitative score ranging from (–) for no positive cells to (++++) for >75% positive cells was used in order to show a prevalent pattern of staining.

## Results

The results are displayed in Table 1. The expression of tumour markers was variable in most cases of CIS, but particularly in specimens from sites adjacent to the combined tumours. The relative number of positive cells given for each case in Table 1 reflects an average percentage in the whole section, but differed markedly from field to field, or even from tubule to tubule. Therefore, a summary of the results allowing an overall view of the prevalent pattern is provided in Table 2.

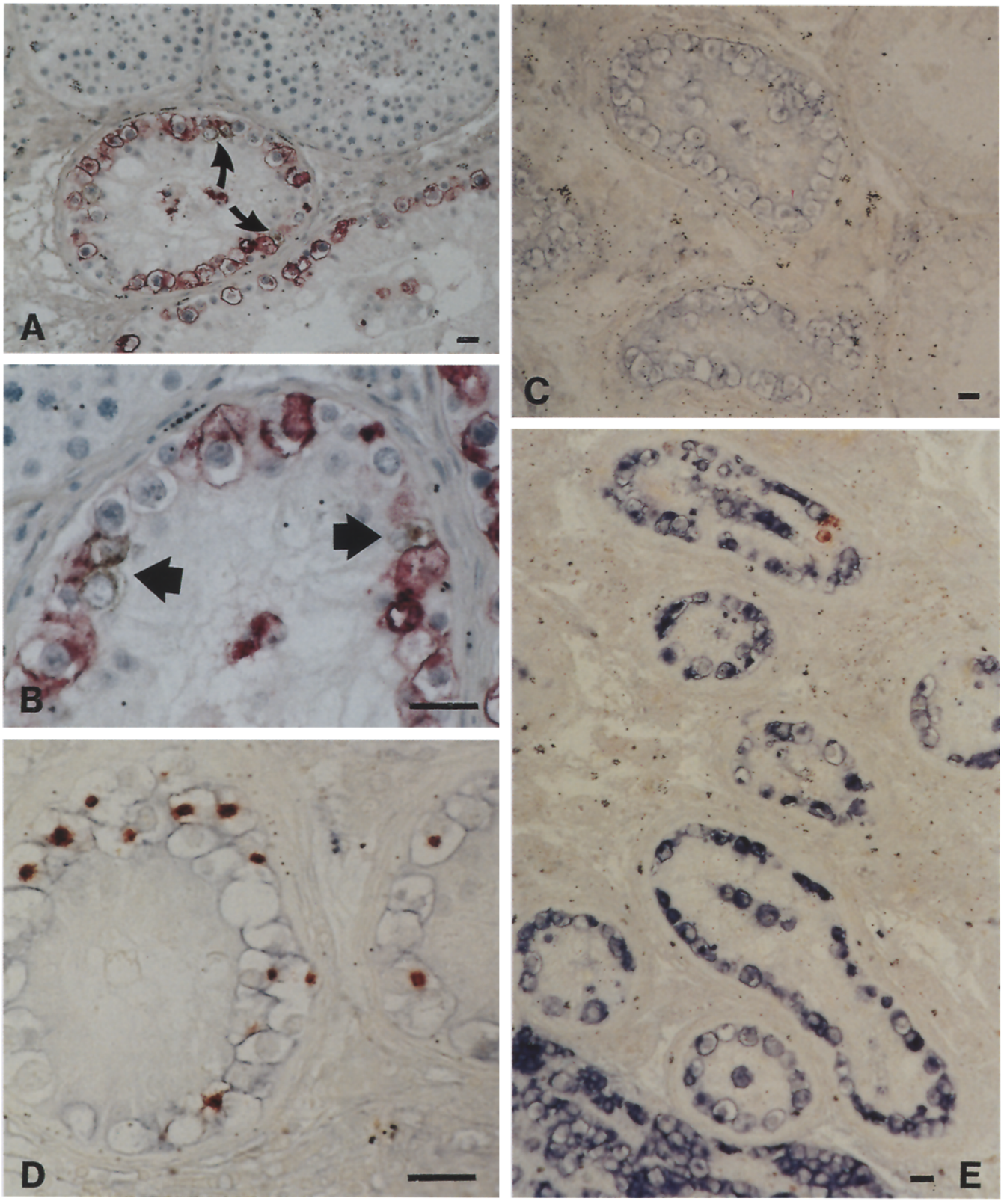
CIS cells present in the vicinity of seminomas expressed mostly Kit and PLAP, with very few cells positive for TRA-1–60 (Fig. 1E), although the adjacent overt seminomas expressed TRA-1–60 to varying degrees. In some cases there were large areas of seminoma that were negative for TRA, with TRA-positive areas in other parts of the same tumour, while in other cases TRA-positive and TRA-negative cells were intimately admixed in nearly equal numbers. The only exception was case 8 (anaplastic seminoma), in which most of the CIS cells and seminoma cells were strongly TRA positive. Interestingly, in many cases, some TRA-positive cells did not express PLAP or Kit (these cells are described in Table 1 as exclusively TRA positive). When CIS was found in the vicinity of a nonseminoma or a combined tumour, the proportion of TRA-positive cells was much greater, in some tubules nearly 100%. However, in the combined tumours marked differences in the expression of the antigens were found among adjacent CIS cells, even those located within the same or neighbouring seminiferous tubules (Fig. 2). In one case (case 13 in Table 1), an unusual pattern of TRA staining was observed. At first glance, the CIS cells expressed only PLAP and Kit, but closer examination revealed the presence of discrete intracellular staining for TRA-1–60 in many cells; this may reflect an early stage of protein expression, or alternatively, an intracellular protein variant might have been recognized by the antibody (Fig. 1E). In the patients with CIS who had not yet developed an overt tumour, the pattern of expression of tumour markers was essentially similar to that of CIS adjacent to seminoma, although the relative number of TRA-positive cells ranged from zero (Fig. 1C) to approximately 2% (Fig. 1A,B).

## Discussion

In this study we provide evidence for the antigenic heterogeneity of the phenotype of CIS cells, despite their morphological uniformity. The morphology of CIS cells adjacent to seminomas and of those located near nonseminomatous tumours was identical, but the pattern of expression of cell surface markers differed markedly. The number of CIS cells expressing TRA-1–60 was usually lower when the lesion was adjacent to a pure seminoma; the opposite trend was observed for Kit and PLAP (Table 2). In combined tumours, the picture was more complicated; in many cases, neighbouring tubules contained CIS cells expressing predominantly different markers (e.g. TRA-1–60 in one tubule and Kit in the next; Fig. 2A). In one case of highly disseminated CIS adjacent to a combined tumour in which microinvasion of interstitial areas by small groups of early seminoma cells could be seen, the neoplastic cells inside and outside the tubule looked alike, but there was a striking difference in the expression of tumour markers (Fig. 2C).

We believe that these observations may be relevant to the discussion on the pathogenetic relationship between CIS and overt tumours. The remarkable phenotypic heterogeneity of CIS cells shown in this study is consistent with the independent tumour progression model, which assumes the ability of CIS cells to transform into both seminoma and nonseminoma. Further linear progression of seminoma into nonseminoma is less likely but cannot be excluded. A modified mixed model of germ cell tumour progression is depicted schematically in Fig. 3.

According to this model, CIS cells originate from primordial germ cells (PGC) and retain their pluripotentiality. At a yet unknown time point, which may be during fetal life, infancy, or puberty, CIS cells undergo further evolution into phenotypes closer to seminoma (CIS-SE) or nonseminoma (CIS-NS). A partial phenotypic difference without obvious changes in morphology is consistent with recent studies conducted by Oosterhuis and his group, who performed cytogenetic analysis of CIS and its invasive counterparts from the perspective of tumour progression. They found that in pure tumours the numbers of copies of chromosomes 15 and 12 were significantly higher in CIS-SE than those in CIS-NS [21, 30]. They suggested the possibility that CIS-SE could make a phenotypic switch into CIS-NS [30]. In line with this reasoning, the progression of seminoma – which itself demonstrates heterogeneity of expression of tumour markers – into nonseminomas cannot be excluded in combined tumours. Clinical experience, however, is strongly in favour of the direct transformation of CIS cells into tumours in nonseminomas. These tumours arise in younger men than do pure seminomas and are


A
B
C
D
E

rather unlikely to develop through any intermediate stages. A recent analysis of glycoforms of a tumour-specific glycoprotein showed that seminoma expressed the gp230 glycoform, which is associated with terminal differentiation, whereas nonseminomatous tumours contained gp200, an embryonic type of glycoform [13]. As double change of the expression of the same molecule including dedifferentiation is rather unlikely, this finding supports independent origins of seminomas and nonseminomas.

In the combined germ cell tumours, the group of Oosterhuis demonstrated that the copy number of chromosome 15 could be either low or high in both components and adjacent CIS cells [8]. This finding suggested that a loss of chromosome 15 was not pathognomonic for nonseminomas and that in most cases, combined tumours had a monoclonal origin. In view of our observation of the heterogeneity of CIS cells, the presence of combined tumours can be explained in most cases by malignant transformation of one or more CIS cells into a seminoma and another CIS cell (or a number of cells) into a nonseminoma.

Recently, on the basis of their studies of genomic imprinting in germ cell tumours, the Dutch researchers have proposed an interesting model that also combines elements of both independent and linear models of tumour progression by assuming separate cells of origin for histological subtypes, which retain the potential for further linear progression. According to their model, CIS may be derived from primitive germ cells that contain nonequivalent paternal and maternal sets of chromosomes, either at an early stage (pre-erased imprinting) or at a later stage of development (post-erased imprinting) [15]. The difference in developmental potential of CIS cells could be manifested only at the invasive stage [20]. Our observation supports this hypothesis. Phenotypic heterogeneity of CIS cells could reflect the initiation of neoplastic process at various stages of differentiation of primordial germ cells. The phenotype of the overt malignant tumour would then depend on the stage at which neoplastic transformation occurred.

Another important observation of this study concerns the expression of TRA-1–60, the antigen present in CIS

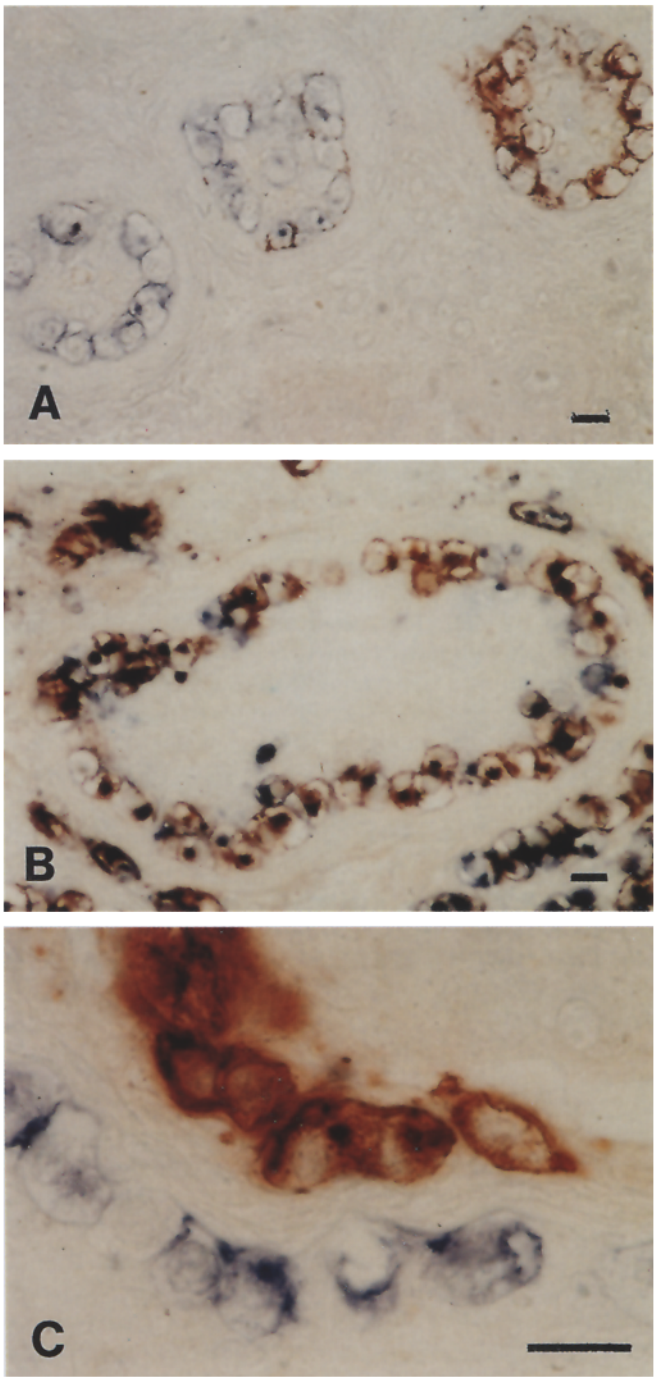



**Fig. 1A–E** Examples of double staining of tumour markers placental-like alkaline phosphatase (PLAP), c-*kit* proto-oncogene protein product (Kit), TRA-1–60 in various specimens of testicular carcinoma in situ (CIS). *Bar* 20 μm. **A** Specimen of isolated CIS (without any invasive tumour). Note strong staining for PLAP (*dark pink*) and weak expression of TRA-1–60 (*brown*; positive cells marked with *arrows*). Tubules with partially preserved spermatogenesis are negative for both markers. **B** Higher magnification of the same specimen. Note the presence of CIS cells expressing TRA-1–60 but not PLAP (*arrows*). **C** Double staining for Kit (*blue*) and TRA-1–60 (*red*) in another case of isolated CIS. There is no detectable expression of the TRA-1–60 antigen. **D** CIS tubules adjacent to a combined tumour (embryonal carcinoma and teratoma with focal seminoma). Most of the cells express Kit (*blue*) and approx. 30% also exhibit unusual intracellular TRA-1–60 staining (*red*). **E** CIS and adjacent pure seminoma shown at the bottom of the picture. Very strong staining for PLAP (*blue*); only one cell positive for TRA-1–60 (*red*)

**Fig. 2A–C** CIS found near combined tumours. Double staining with PLAP (*blue*) and TRA-1–60 (*red*). *Bar* 20 μm. **A** Three tubules with CIS. Tubule on the *left* side of the picture filled with mostly PLAP-positive cells, tubule to the *right*, with predominantly TRA-1–60-positive cells, and tubule in the middle shows an intermediate stage. **B** Most of the cells inside and outside of the tubule (CIS and early seminoma) express both markers to a variable degree. **C** PLAP (*blue*) is expressed by the intratubular CIS only, while morphologically similar early seminoma cells infiltrating the interstitial area outside the same tubule are negative for PLAP but strongly positive for TRA-1–60 (*red*)

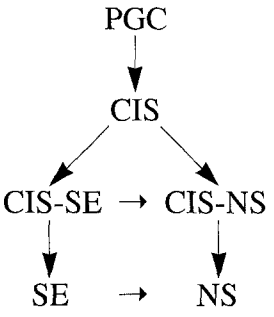



**Fig. 3** Schematic illustration of a modified mixed model of tumour progression

cells [10], which is typical for embryonal carcinoma cells [1] and primordial germ cells [18]. The relative number of CIS cells expressing TRA-1–60 was markedly higher if the lesion was found in the vicinity of nonseminomas or combined tumours. The only exception was case 8, a seminoma with CIS in which approximately 90% of CIS cells were positive for TRA-1–60. However, in this case, the tumour was atypical and was described by pathologists as an anaplastic seminoma, which is a histological form with greater pleomorphism and high mitotic activity. CIS-like malignant tumour cells (early seminoma) microinvading the interstitial areas were much more likely to express TRA-1–60 than the tumour cells still in situ. Thus, TRA-1–60 seems to be expressed predominantly by CIS cells that are "predestined" to tend in the direction of nonseminomas (CIS-NS) and have stronger invasive potential. Therefore, the relative number of TRA-positive cells in patients with isolated CIS who have not yet developed an overt tumour may have prognostic value. Another investigation performed in our laboratory provided additional evidence that CIS-NS may have a more malignant phenotype than CIS found in pure seminomas. The mean area of silver-stained proteins associated with the nucleolar organizer regions of chromososomes (AgNORs), which have been associated with proliferative activity in various types of solid tumours [7, 40], was significantly larger in CIS-NS than in CIS-SE [24].

In conclusion, we have provided evidence that CIS cells are phenotypically heterogeneous. Despite similar morphology and close spatial localization, CIS cells may be in various stages of differentiation. The results of this study support the hypothesis that CIS can progress to both seminomatous and nonseminomatous tumours.

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