Reply

Gandini *et al.* (1) claim that the prostate-specific expression of *DD3*^{PCA3} is restricted to exon 4 of the *DD3*^{PCA3} gene. The authors show that reverse transcription-PCR amplification of the *DD3*^{PCA3} transcript using primers specific for exons 1 and 3 also amplified a *DD3*^{PCA3}-specific product in several nonprostate tissues and cell lines. On the basis of the critical analysis of our published data (2, 3), Gandini *et al.* (1) conclude that the use of exons 1–4-specific PCR primers and the use of a probe located in exon 4 of the *DD3*^{PCA3} gene (for Northern blot analysis) would explain our observed prostate (cancer)-specific expression of *DD3*^{PCA3}. The presence of exon 3 in nonprostatic tissues has been suggested to be attributable to alternative splicing mechanisms, omitting exon 4 of the *DD3*^{PCA3} gene.

After our first publication of the *DD3*^{PCA3} gene (2), we now use exactly the same exon 1 to exon 3 primers as being described in the letter by Gandini *et al.* (1). In the past 4 years, we have amplified *DD3*^{PCA3} in many samples using these primers and have never observed nonprostatic expression of *DD3*^{PCA3}. Although it is not clear from the letter how many cycles of PCR amplification Gandini *et al.* (1) performed, we never used >35 rounds of amplification. We cannot exclude that using more rounds of amplification low levels of expression will be detected. These levels of expression would be far below those observed in prostate cancer, normal prostate, and even prostate cancer cell lines. In addition, we also have performed Northern blot analysis using *DD3*^{PCA3} exon 3-specific probes, resulting in exactly the same prostate cancer-specific expression profiles as shown before (2). These data strongly argue against the alternative splicing of exon 3 in nonprostatic cells.

The former conclusion is further substantiated by our finding that an alternative exon 3 to 4 *DD3*^{PCA3} variant exists in nonprostatic cells (Fig. 1). The level of expression of this product is lower than in normal prostatic tissue and far below the expression in prostate cancer tissue. Strikingly, the *DD3*^{PCA3} variant in nonprostatic tissues is not spliced unlike in prostate-derived samples, as determined by DNA sequence analysis of the PCR products. In normal prostatic tissue the nonspliced transcript is also expressed, although at low levels. In prostate tumor tissue, the nonspliced variant is not expressed or not detectable because of the high overexpression of the spliced *DD3*^{PCA3} product that most probably will be preferentially amplified in the PCR reactions. In RNA samples not subjected to reverse transcription, no amplification product was found (data not shown), indicating that the nonspliced *DD3*^{PCA3} PCR products were not attributable to DNA contamination.

Several explanations for the presence of nonspliced *DD3*^{PCA3} transcripts can be postulated (Fig. 2). Firstly, in prostatic tissues, the *DD3*^{PCA3} transcript may be tissue specifically spliced, a phenomenon that has been described for several other genes (4). Secondly, an alternative ubiquitous promoter may exist in the *DD3*^{PCA3} gene, resulting in a second transcript that is not prostate specific. This option seems less likely, because that transcript seems to be nonspliced despite the strong splice consensus sequences flanking the *DD3*^{PCA3} exons (2). Thirdly, a ubiquitous antisense promoter may be present at the 3′ end of the *DD3*^{PCA3} gene, leading to antisense *DD3*^{PCA3} transcription in many human tissues. It has recently been reported that antisense transcription occurs widespread in the human genome (5),

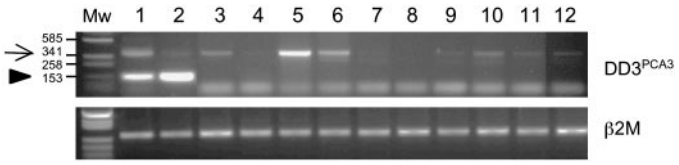

Fig. 1. Expression of *DD3*^{PCA3} in several human tissues using 32 cycles of *DD3*^{PCA3}-specific reverse transcription-PCR with the following primers: forward 5′CAGGAAGCACAAAAGGAAGC-3′ (exon 3, position 443–462) and reverse 5′-TCCTGCCCATCCTTTAAGG-3′ (exon 4, position 593–575). The following tissues have been analyzed: normal prostate (*Lane 1*), prostate cancer (*Lane 2*), heart (*Lane 3*), lung (*Lane 4*), artery (*Lane 5*), kidney (*Lane 6*), liver (*Lane 7*), breast cancer (*Lane 8*), normal breast (*Lane 9*), cervix (*Lane 10*), endometrium (*Lane 11*), and testis (*Lane 12*). The *arrowhead* indicates the spliced *DD3*^{PCA3} transcript (151 bp) in prostate samples only and the *arrow* the nonspliced transcript (378 bp) in the other tissues. Note that the signal in *Lane 2* is saturated. A β-microglobulin PCR was performed as a control (*bottom panel*).

and therefore, it is not unlikely that an antisense *DD3*^{PCA3} transcript exists. Such a putative *DD3*^{PCA3} antisense transcript may be involved in the regulation of *DD3*^{PCA3} transcription in prostate cells or *vice versa* in prostate cells the *DD3*^{PCA3} transcript may affect the, thus far unidentified, antisense transcribed gene. Currently, we are investigating whether alternative splicing or alternative transcription initiation mechanisms are responsible for the expression of the nonprostatic *DD3*^{PCA3}-like transcript.

In conclusion, we agree that transcription of the *DD3*^{PCA3} gene or a *DD3*^{PCA3}-like gene is evident in tissues other than the prostate. However, these transcripts are either not spliced or are complementary (*i.e.*, antisense) to the *DD3*^{PCA3} gene. We have never observed spliced *DD3*^{PCA3} variants (*i.e.*, exon 1–3 product) in nonprostatic tissues. For the application of *DD3*^{PCA3} as a marker for prostate cancer this has one major implication: primers for the amplification of the *DD3*^{PCA3} transcripts in patient samples should cross the large (16 kb) first intron. This region of the *DD3*^{PCA3} gene may be present in the alternative nonspliced or antisense transcripts but is lacking from the prostate-specific spliced form of *DD3*^{PCA3}. Therefore, using exon 1 to exon 3 or 4 primer pairs, only the prostate-specific spliced form of *DD3*^{PCA3} can be amplified (the large intron prevents amplification of this region in the nonspliced transcripts). We have developed two independent assays for the detection of *DD3*^{PCA3} mRNA in patient material, using an exon 1 forward and an exon 4 reverse primer and exon 4-specific detection probes (3, 6). The *DD3*^{PCA3} detection assays have now been applied on >200 patient samples and have been shown to be very specific and sensitive with a strong negative predictive value (6). Furthermore, the analysis of >100 control samples has never revealed any expression of *DD3*^{PCA3} outside the prostate.

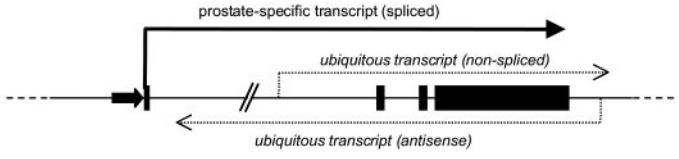

Fig. 2. Schematic representation of the *DD3*^{PCA3} transcription unit. *Boxes* indicate the four DD3^{PCA3} exons and the *solid arrowhead* of the prostate-specific *DD3*^{PCA3} promoter. The *arrows* indicate the different (putative) *DD3*^{PCA3} (-like) transcripts; transcription start sites of the putative ubiquitous transcripts are not determined and are therefore arbitrarily chosen.

Received 4/29/03; accepted 5/21/03.

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