

Original Paper

# Overexpression and gene amplification of *BAG-1L* in hormone-refractory prostate cancer

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## Abstract

**BAG-1L (Bcl-2-associated anthanogene 1) has been found to interact with androgen receptor (AR), and has been suggested to be involved in the development of prostate cancer. In order to determine the presence of genetic and/or expression alterations of BAG-1L in prostate cancer, we analysed human prostate cancer cell lines and xenografts as well as patient samples of untreated, hormone-naïve, and hormone-refractory prostate carcinomas for sequence variations using denaturing high-performance liquid chromatography (DHPLC), for gene copy number using fluorescence *in situ* hybridization (FISH), and for expression using both quantitative RT-PCR and immunostaining. Only one sequence variation was found in all 37 cell lines and xenografts analysed. BAG-1 gene amplification was detected in two xenografts. In addition, gene amplification was found in 6 of 81 (7.4%) hormone-refractory clinical tumours, whereas no amplification was found in any of the 130 untreated tumours analysed. Additionally, gain of the BAG-1 gene was observed in 27.2% of the hormone-refractory tumours and in 18.5% of the untreated carcinomas. In a set of 263 patient samples, BAG-1L protein expression was significantly higher in hormone-refractory tumours than in primary tumours ( $p = 0.002$ ). Altogether, these data suggest that amplification and overexpression of BAG-1L may be involved in the progression of prostate cancer.**

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## Introduction

Androgen and androgen receptor (AR) mediated signalling plays a key role in the development and progression of prostate cancer [1]. It has been known for more than half a century that the growth of prostate cancer is initially androgen dependent [2]. It has also been shown that some of the androgen regulated genes are up-regulated during the emergence of hormone-refractory disease [3]. In addition, it has been demonstrated that overexpression of the *AR* gene [4], which is partly explained by gene amplification [5,6], is in part responsible for converting growth of prostate cancer from androgen dependence to independence.

The AR is a member of the steroid hormone receptor transcription factor superfamily. It activates expression of target genes and gene networks by facilitating

transcriptional initiation. In general, AR mediated transcription requires several auxiliary proteins, so called AR coregulators [1]. Large numbers of AR are coregulators that either enhance or repress the transactivation potential of AR [7]. It has been suggested that alterations in the AR coregulators might be involved in the reactivation of AR signalling during the emergence of hormone-refractory disease [7].

BAG-1 (Bcl-2-associated anthanogene 1) is a multifunctional protein family that associates with the molecular chaperone HSP70. The BAG-1 protein family consists of four different isoforms with molecular masses of ~50 (BAG-1L), ~46 (BAG-1M/RAP46/HAP46), ~36 (BAG-1/p33/p36), and ~29 (BAG-1S/p29) kD. All isoforms are encoded by one *BAG-1* gene, located at chromosome 9p13. Isoforms arise by alternative translation initiation and are localized

differentially inside the cell [8–11]. Interestingly, the longest of the isoforms, BAG-1L, starts with a non-AUG initiation codon, CUG, upstream of the first in-frame methionine [8].

BAG-1 interacts with several types of protein, such as BCL-2, RAF-1, JNK1, and growth factor receptors, such as HGFR and PDGFR [12–14]. BAG-1 has also been found to interact with the nuclear hormone receptor family, including androgen and vitamin D receptors, and to enhance their function [10,15,16]. BAG-1L is the isoform most capable of enhancing the transcriptional activity of the AR. The isoform BAG-1M also interacts with AR, but not as efficiently [17]. In potentiating AR activity, nuclear targeting of the cytoplasmic BAG-1L is required [10,18]. Also, HSP70 is involved in the nuclear action of BAG-1L. It has been shown that the three proteins, AR, HSP70, and BAG-1L, are all located in the androgen response elements of the prostate-specific antigen (*PSA*) gene, and their co-operation enhances the transactivation capacity of the AR [17]. As a consequence, BAG-1L might be involved in the development and progression of prostate cancer [10,19,20].

Here, our aim was to determine whether there are genetic or expression alterations of BAG-1L in prostate cancer that would indicate its involvement in the disease.

## Materials and methods

### Prostate cancer cell lines, xenografts, and clinical material

The prostate cancer cell lines LNCaP, DU-145, PC-3 and 22Rv1 were obtained from the American Type Culture Collection (Rockville, MD, USA), and LAPC4 was kindly provided by Dr C Sawyers (UCLA, Los Angeles, CA, USA). Nineteen LuCaP, and 13 PC human prostate cancer xenografts were made available by two of the authors (RLV and WvW, respectively). Total RNAs from the cell lines and xenografts were isolated using Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) or with Qiagen RNeasy MiniKit (Qiagen Inc, Valencia, CA, USA). The first strand cDNA was synthesized with a Superscript II reverse transcriptase and oligo d(T) primer (Invitrogen Corporation).

The use of clinical tumour materials was approved by the ethics committee of the Tampere University Hospital (TAUH) as well as the National Authority for Medicolegal Affairs. For Q-RT-PCR, freshly frozen clinical specimens of benign prostate hyperplasia ( $n = 9$ ), untreated ( $n = 30$ ) and hormone-refractory ( $n = 12$ ) prostate cancers were obtained from the TAUH (Tampere, Finland). The hormone-refractory samples were from patients who had experienced local progression of the disease during hormonal therapy. The treatment modalities were: four orchiectomy, four

luteinizing hormone-releasing hormone (LHRH) analogue, one oestrogen, two combined androgen blockade (CAB), and one unknown. The median time from the onset of the androgen ablation to progression was 38 months (range: 15–68). Total RNA was isolated and reverse transcribed as previously described [6].

Tissue microarrays (TMAs) were constructed from retrospective formalin-fixed, paraffin-embedded samples of 190 untreated prostatectomy specimens from consecutively treated patients and 81 hormone-refractory transurethral resection specimens obtained from the TAUH. The prostatectomy specimens were re-graded for Gleason score by a uropathologist (PMM). Other clinical data were retrieved from patient files. The median follow-up time of the patients was 47.5 months (range 5–160). The treatment modalities in the group of hormone-refractory patients were: 43 orchiectomy, 24 LHRH analogue, three oestrogen, one bicalutamide, one orchiectomy + oestrogen, eight CAB, and one CAB + estramustine. The median time between diagnosis and progression was 72 months (range 12–180).

### Denaturing high-performance liquid chromatography (DHPLC)

For DHPLC analysis, fragments covering the whole coding area as well as a part of the 5'- and 3'-untranslated regions (UTRs) of the *BAG-1L* (nucleotides 42–1284 from the beginning of transcription according to GenBank Acc. No NM\_003743) gene were PCR amplified from cDNA using Phusion™ High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland). Table 1 shows the primer sequences and annealing temperatures used. PCR products were mixed with the control DNA and subjected to denaturation followed by cooling to 65 °C for 30 min. Owing to high GC content in the region amplified for BAG1s1 and BAG1as1, the PCR was performed using Advantage®-GC Genomic PCR Kit and Advantage®-GC Genomic Polymerase Mix (Clontech laboratories, Inc, Mountain View, CA, USA). The fragments were then directly sequenced.

DHPLC was performed using an Agilent 1100 LC HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a Varian CP28353 Helix DNA Column (50 × 3.0 mm) (Varian Inc, Palo Alto, CA, USA), as previously described [21]. The melting temperatures, and gradients (Table 1) were obtained from the Stanford melt algorithm (<http://insertion.stanford.edu/melt.html> [18 April 2007]), and further adjusted by testing.

### Sequencing

All samples showing more than one peak (or an abnormal peak) in DHPLC were re-amplified, purified, and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM® 3100 sequencer (Applied Biosystems).

**Table 1.** Primers and annealing temperatures for PCR, as well as temperatures and gradient used in DHPLC

Name	Sequence	PCR annealing temperature	DHPLC run temperature	Gradient
1 BAG1s1	ctggcggtcaacaagtg	58	direct	
BAG1as1	cctcactcagggtcaactcc		sequencing	
2 BAG1s2	ttgacctgagtgaggaagc	57	62	57–69
BAG1as2	ccaggcttggacaactggt		63	54–66
3 BAG1s3	agcaatgagaagcacgacct	57	60	56–68
BAG1as3	gagcttcagcttgcaaatcc		61	56–68
4 BAG1s4	gctgaccagctggaagagtt	57	60	56–68
BAG1as4	gccagggaagttgtaga		61	54–66
5 BAG1s5	gattgaaaaggaaggcttgg	62	61	52–64
BAG1as5	ggagcttactcaaatcaaaaga		62	51–63

DHPLC = denaturing high-performance liquid chromatography.

### Q-RT-PCR

The expression of *BAG-1* and the housekeeping gene *TATA-box binding protein (TBP)* was analysed using Q-RT-PCR according to previously described guidelines [6]. Briefly, PCR reactions were performed using the LightCycler™ apparatus (Roche Diagnostics, Mannheim, Germany) with either the LC Fast Start DNA SYBR Green I Kit (*BAG-1*) or the LC DNA Master Hybridization Probes (*TBP*) (Roche Diagnostics). To ensure the specificity of the PCR product, a melting curve analysis was performed, and all PCR products were analysed in a 1.5% agarose gel. Standard curves were prepared from human liver total RNA (Clontech Laboratories Inc, CA, USA).

### Fluorescence *in situ* hybridization (FISH)

For gene copy number analysis, human genomic BAC clones for *BAG-1* (GenBank Acc. No AQ538767) and for chromosome 9 centromere (pMR9A) were labelled by nick translation with digoxigenin-dUTP (Roche Diagnostics) and FITC-dUTP (NEN, Boston, MA, USA), respectively. Dual-colour hybridization to cell lines and xenografts was performed as previously described [6]. For the hybridization to TMAs, the slides were deparaffinized and subsequently cooked for 2 min in an autoclave in 0.05 M Tris-HCl -0.01 M EDTA (pH 9) buffer, and treated with pepsin (Digest-all™, Zymed® Laboratories, San Francisco, CA, USA) in a humid chamber at 37 °C for 20 min, followed by dehydration before hybridization. The FISH signals were scored from non-overlapping and intact nuclei using an Olympus BX50 epifluorescence microscope (Tokyo, Japan). Amplification of the gene was defined as greater than two-fold higher copy number of *BAG-1* than reference probe (9 centromere), or more than five copies of *BAG-1*, or tight clusters of *BAG-1* signals.

### Immunohistochemistry

Immunostaining was performed using the previously described [22] polyclonal rabbit antibody specific for the BAG-1L isoform (N15). The TMA sections were first deparaffinized, followed by antigen

retrieval (autoclave cooking in 10 mM sodium citrate buffer, pH 6.0, at 121 °C for 2 min). The antibody was diluted 1:4 in PowerVision blocking solution (ImmunoVision Technologies Corporation, Brisbane, CA, USA). After incubation and washes, the bound antibody was visualized with PowerVision+™ Poly-HRP IHC Detection Kit (ImmunoVision Technologies Corp.). Sections were counterstained with haematoxylin. Nuclear and cytoplasmic stainings were scored separately from 0 to 3 (0 no staining, 1 weak, 2 moderate, and 3 strong staining) independently by two of the authors (HEM and TV) in a blinded fashion. Subsequently, the mean score was calculated. The mean score 0–1.5 indicated no to weak, and 2–3 moderate to strong staining. Staining without primary antibody was used as a negative control.

### Statistical analysis

Fisher's exact test and a  $\chi^2$ -test were used to evaluate the association of BAG-1L protein expression and gene copy number with different clinicopathological variables. Non-parametric Mann–Whitney *U* and Kruskal–Wallis tests were used for association analysis of Q-RT-PCR data. Progression-free survival analysis was done using the Kaplan–Meier method together with the log rank test.

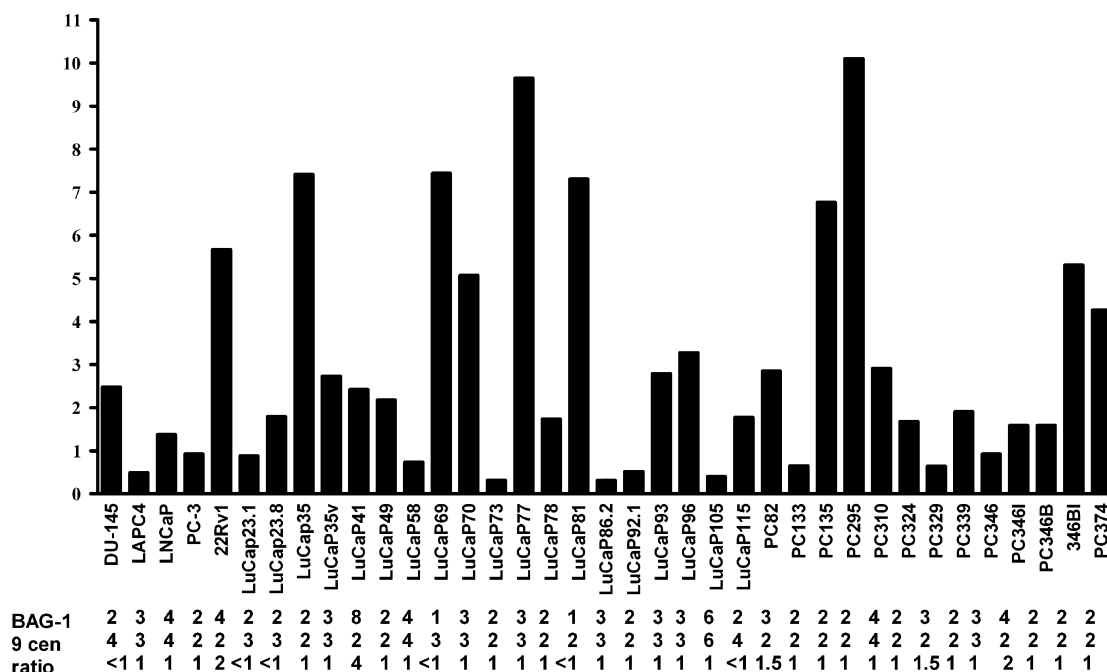
## Results

### Mutation screening

All five prostate cell lines and 32 xenograft samples were screened for mutations of the coding area of *BAG-1L*, by DHPLC and sequencing. One sequence variation, insertion of C at base 1141 in the 3'-UTR (starting from the initiation of translation according to GenBank Acc. No NM\_004323), was found in xenograft PC-310.

### Gene copy number analysis

Five prostate cell lines, 32 xenografts, and 211 clinical tumours were successfully screened for gene copy



**Figure 1.** The relative expression of *BAG-1* in prostate cancer cell lines as well as in PC- and LuCaP xenografts as determined by Q-RT-PCR. The gene copy number of *BAG-1* and chromosome 9 centromere (reference) as well as their ratio is shown

number alterations using FISH. Of the cell lines studied, 22Rv1 showed two signals with the centromere 9 probe and four signals with the *BAG-1* probe. DU-145 had four centromere and two *BAG-1* signals indicating deletion, whereas LNCaP showed four, PC-3 two and LAPC4 three copies of both *BAG-1* and centromere. Of the xenografts, only LuCaP41 and LuCaP105 (6.2% of the xenografts) showed amplification of the *BAG-1* gene. In addition, 12 (37.5%) other xenografts showed gain (additional copies) of the *BAG-1* gene (Figure 1).

Of the 130 untreated prostate tumours analysed, none showed amplifications of the *BAG-1* gene, whereas 24 (18.5%) showed gain, and 3 (2.3%) loss of *BAG-1* gene (Table 2). Of the 81 hormone-refractory tumours, 6 (7.4%) showed amplification, 22 (27.2%) gain, and 1 (1.2%) loss. The frequency of amplification was significantly ( $p = 0.0028$ , Fisher's exact test) higher in hormone-refractory than in untreated tumours.

**Table 2.** Gene copy number alteration of *BAG-1* in clinical prostate tumours

	<i>BAG-1</i> gene copy number			<i>p</i> -value <sup>†</sup>
	Non-amplified	Gained	Amplified*	
Untreated	106 (81.5)	24 (18.5)	0 (0)	0.0014
Hormone-refractory	53 (65.4)	22 (27.2)	6 (7.4)	

Results are shown as No (%).

\* Amplification was defined as greater than two-fold higher copy number of *BAG-1* than chromosome 9 centromere, or more than five copies of *BAG-1*, or tight clusters of *BAG-1* signals.

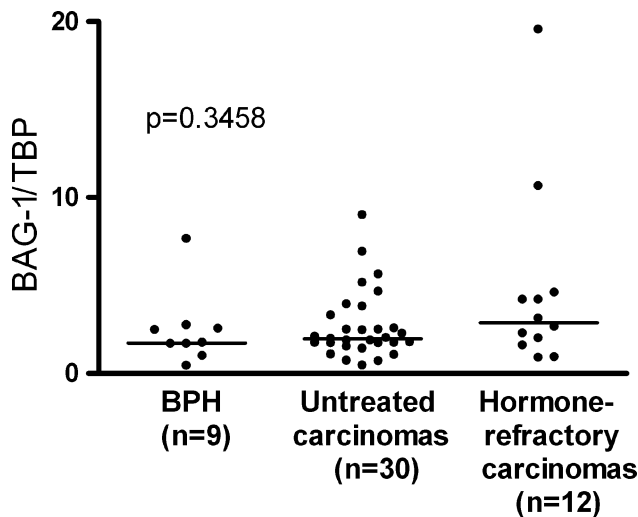
<sup>†</sup>  $\chi^2$ -Test

### mRNA expression analysis by Q-RT-PCR

In the five cell lines and 32 xenografts studied, relative mRNA expression levels varied greatly (Figure 1). Of the cell lines, 22Rv1, which was the only cell line showing increased *BAG-1* to chromosome 9 centromere copy number ratio, had the highest expression of *BAG-1*. Also, mRNA levels from nine benign prostatic hyperplasia (BPH), 30 primary prostate cancers and 12 hormone-refractory samples were measured (Figure 2). Median mRNA expression values for relative *BAG-1/TBP* ratios were 1.65- and 1.46-fold higher in hormone-refractory tumours than in BPH or untreated tumours, respectively. However, the differences were not statistically significant ( $p = 0.3458$ , Kruskal–Wallis test).

### Protein expression by immunohistochemistry

TMA's containing 263 clinical tumour samples of untreated ( $n = 190$ ) and hormone-refractory ( $n = 73$ ) prostate carcinomas were successfully stained (Table 3). The *BAG-1L* isoform specific antibody stained predominantly nuclei of cancer and normal glandular epithelial cells (Figure 3). However, frequently, weak staining in the cytoplasm was also seen. Of the untreated primary tumours, the nuclear staining was negative or weak (score 0–1.5) in 134 (70%), and moderate or strong (score 2–3) in 56 (30%) cases. Nuclear staining was negative or weak in 36 (49%), and moderate or strong in 37 (51%) of the hormone-refractory tumours. Overall, hormone-refractory tumours showed significantly ( $p = 0.0016$ ) stronger expression of *BAG-1L* than untreated tumours (Table 3). Cytoplasmic staining in untreated tumours was negative or weak in 155 (82%), and



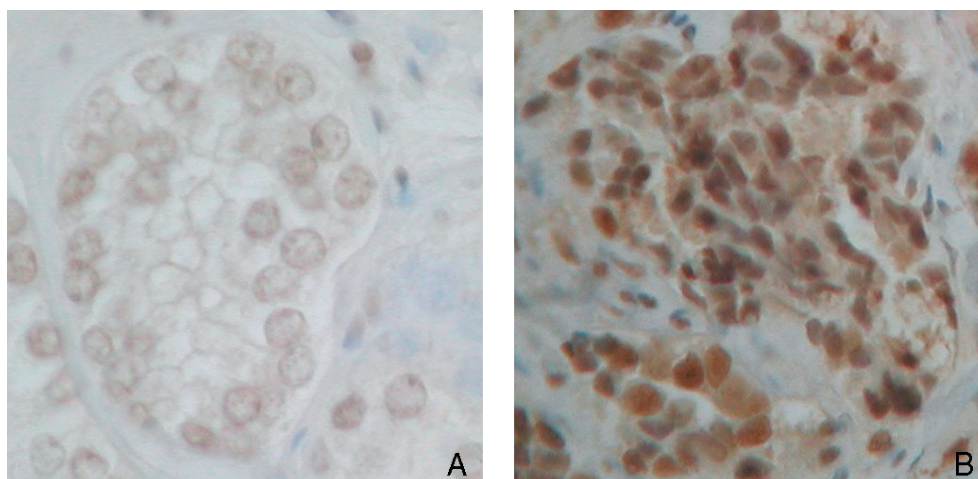
**Figure 2.** The relative expression of *BAG-1* from clinical tumour samples was determined by Q-RT-PCR. Median expression values for relative *BAG-1/TBP* ratios were  $\times 1.65$ - and  $\times 1.46$ -fold higher in hormone-refractory tumours than in BPH or untreated tumours, respectively. BPH = benign prostatic hyperplasia

moderate in 35 (18%) cases, whereas 55 (75%) of the hormone-refractory tumours were negatively or

weakly stained, and 18 (25%) showed moderate staining ( $p = 0.3031$ ) (Table 3). In untreated prostate cancer, neither nuclear nor cytoplasmic staining was associated with Gleason score ( $p = 0.7137$  and  $p = 0.3534$ , respectively), pT-stage ( $p = 0.7273$  and  $p = 0.1536$ , respectively) (Table 3) or progression-free survival ( $p = 0.1073$  and  $p = 0.8318$ , respectively).

## Discussion

Using a BAG-1L isoform-specific antibody, we have shown for the first time that nuclear expression of BAG-1L is increased in locally recurrent hormone-refractory prostate cancers compared with untreated primary prostate tumours. Of all the BAG-1 isoforms, BAG-1L has previously been shown to be the most potent AR co-activator [17]. In the patients treated by prostatectomy, there was no association between nuclear BAG-1L staining and Gleason score, pT-stage or progression-free survival. This may indicate that the up-regulation of BAG-1L is specifically associated with tumour progression to a hormone-refractory state. Krajewska and co-workers [20] have previously shown that nuclear BAG-1 expression is increased in



**Figure 3.** An example of (A) BAG-1L immunonegative, and (B) BAG-1L immunopositive clinical prostate carcinoma

**Table 3.** Association of clinicopathological variables with expression of the BAG-1L protein in prostate cancer

	Nuclear staining			Cytoplasmic staining		
	0–1.5	2–3	p-value	0–1.5	2–3	p-value
Untreated	134 (70.5)	56 (29.5)		155 (81.6)	35 (18.4)	
Hormone-refractory	36 (49.3)	37 (50.7)	0.0016	55 (75.3)	18 (24.7)	0.3031
Untreated:						
Gleason score:						
<7	55 (71.4)	22 (28.6)		64 (83.1)	13 (16.9)	
= 7	58 (65.9)	30 (34.1)		72 (81.8)	16 (18.2)	
>7	18 (81.8)	4 (18.2)	0.7137	16 (72.7)	6 (27.3)	0.3534
pT-stage:						
pT1 + pT2	93 (69.9)	40 (30.1)		112 (84.2)	21 (15.8)	
pT3	41 (73.2)	15 (26.8)	0.7273	42 (75.0)	14 (25.0)	0.1536

Results are shown as No (%).

prostatectomy specimens that do not show response to neoadjuvant hormonal therapy [20]. Although the antibody used by Krajewska and co-workers recognizes all BAG isoforms, their data may indicate that BAG-1L is overexpressed in primary tumours that are less responsive to androgen withdrawal. Together the data support a role for BAG-1L in the development of hormone-refractory prostate cancer.

The BAG-1L antibody also showed weak staining in the cytoplasm that was not associated with tumour type, Gleason score, stage or prognosis. Krajewska and co-workers [20] reported that the BAG-1 protein level rises in the cytoplasm of prostate cells during early tumour progression. They showed also that high cytosolic BAG-1 was associated with shorter progression-free time in patients treated by radiation therapy. As we studied BAG-1L expression specifically and found no association with survival, it is likely that the cytosolic staining demonstrated by Krajewska and co-workers (2006) is from isoforms other than BAG-1L.

The mRNA expression of *BAG-1* measured by Q-RT-PCR suggested a small, non-significant increase of *BAG-1* expression in hormone-refractory prostate tumours as compared with benign prostate hyperplasia or untreated primary tumours. Since all *BAG-1* isoforms are translated from the same transcript, one cannot design an RT-PCR assay that would detect mRNA expression of specifically *BAG-1L*. This may explain the observed discrepancy between immunohistochemistry and Q-RT-PCR data, as increased expression of BAG-1L in hormone-refractory tumours might be masked in the Q-RT-PCR by the expression of other *BAG-1* isoforms. Alternatively, the overexpression of BAG-1L protein may be due to mechanisms other than mRNA overexpression.

By using array comparative genomic hybridization (aCGH), we have recently shown that chromosomal region 9p13, in which the *BAG-1* gene is located, is gained in one-third of prostate cancer cell lines and xenografts [23]. To follow up on this, we studied the actual copy number of the *BAG-1* gene in our set of samples using FISH. Amplification of the gene could only be identified in LuCaP41 and LuCaP105. These cell lines are both derived from hormone-refractory tumours in individual patients. Similarly, 6/81 (7.4%) of the clinical hormone-refractory tumours showed amplification of the *BAG-1* gene, and 22/81 (27.2%) showed gain of the *BAG-1* gene. Thus, altogether, 34% of the hormone-refractory tumours contained additional copies of the *BAG-1* gene, consistent with our earlier aCGH-based study. None of the untreated tumours showed amplification of the *BAG-1* gene by FISH, with an additional 18.5% showing gain of *BAG-1*.

We have recently narrowed down the minimal region of 9p13 amplification by FISH mapping (Saramäki and Visakorpi, unpublished data). It seems that the *BAG-1* gene is located slightly telomeric of the minimal region of amplification. Thus, it is possible

that *BAG-1* is not the main target gene for the amplification. Also, the finding that LuCaP41 and LuCaP105, which contain the *BAG-1* gene amplification, did not show particularly high expression of *BAG-1* (Figure 1) suggests that *BAG-1* might not be the target of the amplification in these xenografts. However, this finding might also be due to the fact that Q-RT-PCR measures the expression of *BAG-1*, and not specifically *BAG-1L*, as explained above. However, amplicons are typically known to contain several genes and there may be several target genes for the same amplicon [24]. It has also been shown that genes located nearby the primary target of the amplification may be functionally important [25]. Thus, the amplification of *BAG-1*, although not necessarily being the only target gene of the amplification, might well contribute to the emergence of hormone-refractory prostate cancer.

We also screened the whole coding region of the *BAG-1L* gene for sequence variations using heteroduplex analysis with DHPLC. Only one sequence variation in the non-coding region (3'-UTR) was found in one androgen sensitive xenograft (PC310), suggesting that mutations in *BAG-1L* are rare. As the cell lines and xenografts have probably accumulated all possible genetic alterations that exist in prostate cancer, we reasoned that it would be highly unlikely that mutations would be found in clinical tumour specimens. Therefore, they were not analysed further.

It has now been widely recognized that AR mediated signalling is reactivated during the emergence of hormone-refractory prostate cancer [1]. One mechanism seems to be overexpression of AR, which is partly explained by amplification of the *AR* gene. Another suggested mechanism is altered expression of AR coregulators [7]. Indeed, increased expression of, for example, SRC1 and TIF2 [26] has been reported in hormone-refractory prostate cancer. We have recently analysed this same material for SRC1 protein expression [20]. We found a non-significant trend towards increased expression of SRC1 in hormone-refractory tumours. In contrast, we have earlier shown that *SRC1* mRNA expression is actually decreased in hormone-refractory carcinomas [6]. Here, a highly significant overexpression of BAG-1L in the hormone-refractory tumours as compared with untreated carcinomas was demonstrated. This finding supports published experimental data suggesting that BAG-1L may be involved in the emergence of ablation-resistant tumour clones. It has been shown that BAG-1L reduces the concentration of 5 $\alpha$ -dihydrotestosterone required to transactivate AR as well as diminishing the inhibitory effects of antiandrogens [10]. Thus, BAG-1L could facilitate the transactivation of AR during castration and/or antiandrogen treatment.

In conclusion, significantly increased nuclear protein expression of BAG-1L was found in hormone-refractory prostate cancers (30% vs 50%). In addition, amplification of the *BAG-1* gene was found in 7% of the hormone-refractory tumours, with none found

in untreated tumours. Moreover, 27% of hormone-refractory tumours showed gain of *BAG-1* compared with only 18% of the untreated prostate samples. Together, these findings suggest that BAG-1L may be involved in the emergence of hormone-refractory prostate cancer.

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