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High throughput quantitative reverse transcription PCR assays revealing over-expression of cancer testis antigen genes in multiple myeloma stem cell-like side population cells

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Summary

Multiple myeloma (MM) stem cells, proposed to be responsible for the tumourigenesis, drug resistance and recurrence of this disease, are enriched in the cancer stem cell-like side population (SP). Cancer testis antigens (CTA) are attractive targets for immunotherapy because they are widely expressed in cancers but only in limited types of normal tissues. We designed a high throughput assay, which allowed simultaneous relative quantifying expression of 90 CTA genes associated with MM. In the three MM cell lines tested, six CTA genes were over-expressed in two

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Competing interest

The authors declare no competing financial interests.

Author contribution

JW, HL, and WT performed the research. JW, HL, WT, and LK designed the research and the experiments and analysed the data. CC and YZ supervised the research and provided funding. JF and BS discussed the results. JW wrote the manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Immunofluorescence membrane staining with anti-AURKA antibody in SP and MPC of RPMI8226 cells.

Fig S2. Up-regulated CTA genes in the ALDH high population of MM cell line compared with ALDH low population.

Table SI. MM patient information.

Table SII. Up-regulated CTA genes in SP of MM patient BMs.

and *LUZP4* and *ODF1* were universally up-regulated in all three cell lines. Subsequent study of primary bone marrow (BM) from eight MM patients and four healthy donors revealed that 19 CTA genes were up-regulated in SP of MM compared with mature plasma cells. In contrast, only two CTA genes showed a moderate increase in SP cells of healthy BM. Furthermore, knockdown using small interfering RNA (siRNA) revealed that *LUZP4* expression is required for colony-forming ability and drug resistance in MM cells. Our findings indicate that multiple CTA have unique expression profiles in MM SP, suggesting that CTA may serve as targets for immunotherapy that it specific for MM stem cells and which may lead to the long-term cure of MM.

Keywords

multiple myeloma; cancer testis antigen; side population; high throughput; quantitative RT-PCR

Multiple myeloma (MM) is the second most common haematological cancer and represents 10% of all haematopoietic malignancies in the United States (Jemal *et al*, 2007). In 2010, the National Cancer Institute reported 20 180 new cases of MM and 10 650 deaths directly attributed to MM (Cruz *et al*, 2011). Despite recent major improvements in treatment, MM still remains incurable and long-term survival appears elusive (Chanan-Khan *et al*, 2010; Borrello, 2012). Current therapy focuses on killing the myeloma cells with cytotoxic agents, targeting myeloma cell–specific pathways, or inhibiting the myeloma-dependent microenvironment. Although these therapies can lead to initial complete clinical response, the majority of patients develop relapsed disease with resistance to these therapies (Munshi *et al*, 2002; Richardson *et al*, 2003). This has led to the hypothesis that myeloma stem cell-like cells, which have increased resistance to many cytotoxic agents and possess self-renewal capacity, may be responsible for the relapsed disease (Hajek *et al*, 2013). Additional methods are required to eliminate the myeloma stem cells for the long-term cure of MM.

Cancer testis antigens (CTA) are a promising class of tumour antigens for T-cell-mediated immunotherapy due to their limited expression in somatic tissue. An earlier study demonstrated that CTA could be specifically recognized *in vitro* by cytotoxic T-cells (CTLs) in patients with melanoma (van der Bruggen *et al*, 1991). Since then, more and more CTA genes have been characterized and tested as potential targets for cancer therapy (de Carvalho *et al*, 2012; Mengus *et al*, 2013). Furthermore, another recent study showed that CTA genes were highly and frequently expressed in glioma cancer stem cells compared with differentiated cells (Yawata *et al*, 2010). Encouraged by previous reports, we hypothesized that CTA genes may have a different expression profile in myeloma stem cells compared to mature plasma cells (MPC) and the over-expressed CTA genes may become ideal targets for immunotherapy for the elimination of myeloma stem cells.

In present study, based on gene expression data from MM and normal samples, we designed a high throughput quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay to simultaneously measure the expression of 90 CTA genes associated with MM. We identified several CTA genes whose expressions are increased in the MM SP. Furthermore, we found that one of those up-regulated CTA genes, *LUZP4*, is required for colony forming

and drug resistance in MM cells. These findings will lead to the development of new CTL-mediated therapies by targeting these CTA genes, for the benefit of MM patients.

Material and methods

Cell cultures and reagents

Multiple myeloma cell lines RPMI8226, MM1S and U266 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA), 100 u/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Houston, TX, USA), as previously reported (Wen *et al*, 2011).

Bone marrow (BM) aspirates were collected from eight MM patients and four healthy donors under a protocol approved by the Houston Methodist Research Institute and informed consent was obtained, in compliance with the Declaration of Helsinki. Primary cells were purified from freshly isolated BM by Ficoll (MP Biomedicals, Solon, OH, USA) density sedimentation. Cells were cultured in RPMI 1640 medium containing 10% FBS, 100 u/ml penicillin, 100 μg/ml streptomycin and 2 mmol/l-glutamine, and maintained at 37°C in 5% CO_2.

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

SP analysis and cell sorting by flow cytometry

To identify the SP in MM cell lines, cells were seeded at $0{\cdot}5 \times 10^6$ cells/ml in a T75 flask for 3 d (72 h after seeding) in RPMI 1640 medium with 10% FBS, supplemented with 100 u/ml penicillin and 100 μg/ml streptomycin. Cells were harvested and washed in pre-warmed RPMI 1640 medium with 2% FBS and 10 mmol/l HEPES buffer, and then resuspended at a concentration 1×10^6 cells/ml in RPMI 1640 medium with 2% FBS and 10 mmol/l HEPES. Hoechst 33342 water solution (1 mg/ml) was then added to make a final concentration of 5 μg/ml followed by incubation in a water bath at 37°C for 90 min with shaking every 15 min. Immediately after incubation, the samples were put on ice to stop dye efflux and washed with ice-cold Hank's balanced salt solution (HBSS) containing 2% FBS and 10 mmol/l HE-PES. Subsequently, Hoechst-labelled cells were stained with CD138-PE monoclonal antibody (BD Biosciences, San Jose, CA, USA) for 15 min on ice.

To identify the SP in primary samples, cells were harvested and washed in pre-warmed RPMI 1640 medium with 2% FBS and 10 mmol/l HEPES buffer. SP staining was performed as for the cell lines.

Cells were then analysed using FACS Aria (Becton Dickinson, San Jose, CA, USA). The Hoechst 33342 dye was excited at 357 nm and its fluorescent emission was measured at Hoechst blue fluorescence and Hoechst red fluorescence. SP cells and mature plasma cells (i.e., CD138-positive non-SP cells) were then sorted accordingly (Fig 1).

Aldefluor assay by flow cytometry

Aldefluor assay was performed according to the manufacturer's instruction (Stem Cell Technologies, Vancouver, BC, Canada). The aldehyde dehydrogenase (ALDH) high and low populations of RPMI8226 cells were analysed and sorted with the FACS Aria.

cDNA synthesis

cDNA from sorted cells was synthesized with the WT-Ovation RNA Amplification System (Nugen, San Carlos, CA, USA) according to the user's guide. The cDNA concentration was estimated using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

CTA gene selection

Cancer testis antigens expression in various types of cancers should be different. In order to identify CTA signatures in MM among different stage MM patients, we used the open access microarray database: Mayo Clinic MM Microarray. This database was established by analysing BM samples of 91 new, 23 smouldering and 26 relapsed MM patients from MM research consortium (MMRC, http://www.broadinstitute.org/mmgp/home). Then, we integrated this database with the CTA gene bank database (http://www.cta.lncc.br/) to specifically study the expression of CTA genes in MM patients and identified 90 CTA genes expressed by the majority of MM patients (Fig 2A).

Primers and qRT-PCR

We designed primers for these 90 CTA and 5 housekeeping genes (data not shown) using Primer 3 software and selected those that contained at least 1 exon-exon junction to reduce the genomic DNA contamination. One pair of primers for each gene was added to each well of a 96-well plate to form the assay, comprising 90-CTA genes and 5 housekeeping genes, and one well as a blank control without any primer. qRT-PCR analysis of the gene expression was then performed using RT^2-SYBR® Green PCR Master Mix (Qiagen, Valencia, CA, USA) with 40 cycles of 15 s at 95°C and 1 min at 58°C on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Fluorescence data were collected at 58°C after each cycle. After the final cycle, melting curve analysis of all samples was conducted within the range of 58–95°C. The specificity of the PCR products was verified by the targeted product size using gel electrophoresis and melting curve analysis. The threshold cycle and $2^{-\Delta\Delta C_t}$ method were used for calculating the relative amount of the target RNA using the average of the five house-keeping genes as internal control, according to user's manual. The experiments were repeated in triplicate.

Immunoglobulin heavy chain (IGH) gene rearrangement analysis

DNA was isolated from cells using the QIAmp system (Qiagen). The concentration of DNA was estimated using the NanoDrop ND-1000 spectrophotometer. DNA quality was assessed using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). PCR primer sequences for *IGHV* gene framework region 3 (FR3) were designed as previously reported (Kummalue *et al*, 2010). PCR of the *IGH* genes to assess clonality used the BIOMED-2 system. Master mixes were purchased from Invivoscribe Technologies (San Diego, CA,

USA), and the PCR was done as per manufacturer's instructions and used HotStart *Taq* DNA polymerase (Qiagen) (Burack *et al*, 2010).

Immunofluorescence

SP and non-SP (NSP) cells were sorted with Hoechst 33342 staining. After washing, cells were resuspended in phosphate-buffered saline (PBS) and transferred to a coverslip coated with poly-L-lysine. After a 1-h incubation at room temperature, excess cell suspension was removed. Cells were washed, fixed, blocked with PBS containing 0·1% bovine serum albumin, and incubated with primary anti-AURKA antibody (Thermo Fisher Scientific) for 1 h. Subsequently, cells were washed with PBS and incubated with TXRed-labelled secondary antibody. After washing, coverslips were mounted on slides in fluorescent mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (ProLong antifade reagent with DAPI; Invitrogen, Carlsbad, CA, USA). Fluorescence was detected using an Olympus microscope and images were acquired using Cellsens Dimension software.

SiRNA knock down

LUZP4 gene siRNA and scrambled siRNA (Santa Cruz Biotechnology, Dallas, TX, USA) were used at 100 nmol/l to transfect RPMI8226 cells using Lipofectamine RNAiMAX (Invitrogen), as previously reported (Wen *et al*, 2008). Knockdown efficiency of siRNAs was confirmed by Western blot analysis.

Western blotting

Cell lysates were prepared and analysed by Western blot as described previously. The membrane was probed with antibody to LUZP4 (Santa Cruz Biotechnology). After detection, the membrane was completely stripped and ACTB (β-actin) was used as loading control (Wen *et al*, 2008).

Cell colony assay

A soft agar colony assay was performed as previously reported (Wen *et al*, 2011). Briefly, 1·5 ml base agar layers of 0·6% agarose were prepared in 35 mm dishes by combining equal volumes of 1·2% low melting temperature agarose (Thermo Fisher Scientific) and 2× RPMI 1640 medium + 20% FBS + 2× antibiotics. Next, 5×10^3 cells were resuspended in 0·75 ml 2× RPMI 1640 medium + 20% FBS + 2× antibiotics and mixed with 0·75 ml volumes of 0·6% agar, then immediately plated on top of base agar. Complete medium was added on the top and changed twice a week. After 2 weeks, dishes were stained with methylene blue, pictures were taken under a phase contrast microscope, and colony number was counted.

Flow cytometric analysis of apoptosis

Cells were treated with arsenic trioxide (Sigma-Aldrich Co.) or Bortezomib (Millennium Pharmaceuticals, Cambridge, MA, USA) for 24 h. Dual staining with Annexin V-fluorescein isothiocyanate (FITC) and PI (Propidium iodide) (Becton Dickinson) was used to detect apoptosis according to the manufacturer's instructions as previously reported (Wen *et al*, 2008, 2010).

Statistical analysis

Statistical analysis was conducted with the SPSS 10 SPSS Inc., Chicago, IL, USA), using *t*-test or 1-way analysis of variance (ANOVA) where appropriate.

Results

SP cells from myeloma patients containing clonal population

The flow cytometric analysis revealed that in primary MM patient BM, SP cells are CD138-negative and NSP cells are CD138+ (Fig 1A, B). Immunoglobulin heavy chain (*IGH*) rearrangement study showed that SP and MPC from myeloma patients possess the identical *IGH* rearrangement pattern (Fig 1C). This result indicates that the SP of these patients contains myeloma stem cells that are of same cell origin of the mature neoplastic plasma cells.

MM-CTA were enriched in SP in MM cell lines

With the strategy shown in Fig 2A, we designed the MM-specific CTA gene assay composed of 90 CTA genes and 5 housekeeping genes. Among these 90 CTA genes, 5 genes were up-regulated in the new MM patient and 4 genes were up-regulated in the patient with smouldering MM. Futhermore, 17 CTA genes were up-regulated in relapsed MM patient, compared with normal control (Fig 2B). The expression levels as well as the ratio to normal control in relapsed MM, smouldering MM, and new MM are shown in Table I.

To test if CTA gene expression is enriched in MM SP, we investigated the CTA expression profile in various myeloma cell lines and primary samples. We collected SP and MPC, respectively, from MM cells lines including RPMI8226, MM1S and U266. Using a threshold of >2-fold change for genes expression (SP: MPC) and P value < 0·05 after *t*-test, 16 CTA genes were up-regulated in RPMI8226 (Fig 3A), 13 CTA genes in MM1S (Fig 3B) and 11 CTA genes in U266 cell line (Fig 3C). Of note, among these genes, *GAGE12F*, *MAGEA4*, *MAGEA5*, *SSX1*, and *TOP2A* genes showed overlap in 2 of 3 cell lines, and *LUZP4* and *ODF1* were universally up-regulated in all 3 MM cell lines. Furthermore, Immunofluorescence membrane staining with anti-CTA antigen AURKA antibody was performed. In agreement with our PCR data, the AURKA antibody stain showed higher membrane expression in SP cells than in MPC (Figure S1).

MM-CTA were enriched in SP in MM BM samples

We further extended our study to primary MM cells. Nineteen CTA genes were significantly up-regulated in SP (fold change >2 compared with MPC, P < 0·05, *t*-test); Fig 4A. Interestingly, *AURKA*, *DDX43*, *FANCI*, *MAGEA3*, *TEX14*, and *LUZP4* were also identified as up-regulated genes in the SP of at least 1 MM cell line. In addition, 3 CTA genes (*AKAP4*, *MAGEA3*, and *SSX2*) and 7 genes (*ANKRD45*, *FANCI*, *LUZP4*, *MAGEA12*, *MAGEB3*, *RRM2*, *and TEX14*) were over-expressed by the SP of 8/8 and 7/8 MM patients, respectively (Table II). The detailed information for MM patients and fold-change of CTA gene in each patient are shown in Tables SI and SII, respectively.

In contrast to MM BM, only 2 CTA genes, *DUT* and *KDM5B* (*JARID1B*), showed moderate increase (2·2– and 4·5-fold, respectively) in the SP of normal BM compared with MPC (Fig 4B). Of interest, these 2 genes were not up-regulated in the SP of MM patients. Additionally, except for *AKAP4* (3·95-fold), *CFLAR* (2·93-fold), *HIST1H2BG* (2·24-fold), *MAGEB2* (4·18-fold) and *MAGEB3* (3·97-fold), the expression level of 19 up-regulated CTA genes in SP of MM BM was more than 5 times higher than that in SP of normal BM control (Fig 4C).

It should be noted that the SP cells in the myeloma patients include both cancer stem cells and normal haematopoietic stem cells (HSC) while the SP cells in normal samples contain only normal HSC (Morita *et al*, 2006; Golebiewska *et al*, 2011). Thus, the differences of CTA expression between the SP cells of myeloma patients and controls are most probably due to the increased expression of CTA genes of cancer stem cells within the SP cells of myeloma patients.

All these results suggested that CTA have unique expression profiles in the SP of MM cells, suggesting that CTA may serve as targets for immunotherapy that is specific for MM stem cells, which may lead to the long term cure of MM.

LUZP4 gene expression is required for colony formation and drug resistance

To explore the function of the gene *LUZP4*, which was universally up-regulated in SP of MM cell lines and primary MM BM, we used siRNA to knockdown gene expression and the efficiency was confirmed by Western blot (Fig 5A). Given its up-regulated expression level in MM stem cells, which play a critical role in tumour development, disease recurrence, resistance and chemotherapy (Huff & Matsui, 2008; Matsui *et al*, 2008; Brennan & Matsui, 2009; Ghosh & Matsui, 2009; Jakubikova *et al*, 2011; Paino *et al*, 2012), we hypothesized that the function of *LUZP4* may be related to colony forming ability and drug resistance. Firstly, the *LUZP4* siRNA knocked-down U266 cells and control cells were grown on agar for 2 weeks: the results showed that *LUZP4* knocked-down cells formed fewer colonies compared to control cells. Next, we treated the *LUZP4* knocked-down U266 cells with arsenic trioxide and bortezomib for 24 h and the results showed that *LUZP4* knocked-down U266 cells were more sensitive to chemotherapy.

Discussion

Our results have indicated that certain CTA genes (e.g., *AURKA*, *DDX43*, *FANCI*, *MAGEA3*, *TEX14* and *LUZP4*) are upregulated in both SP cells of the majority of MM cell lines and primary marrow samples from MM patients compared to the mature myeloma cells. This finding indicates that upregulation of CTA genes is a common feature for myeloma cells with stem cell features. This is in accordance with previous reports showing that enhanced expression of CTA genes in glioma stem cells and that CTA genes promoters may be tightly regulated by methylations and the methylation levels in promoter regions in cancer stem cells are lower than that in differentiated cells (Yawata *et al*, 2010). This mechanism may have contributed to the up-regulation of CTA in cancer stem cells in general and in myeloma stem cells in our study.

In this study, we used SP cells identified by flow cytometry, based on the unique property of stem cells, which pump out Hoechst 33342 dye due to their high expression of ATP binding cassette transporters, to identify myeloma stem cells in MM. This is because studies have demonstrated that SP cells exhibited clonogenic and tumourigenic potential in MM (Jakubikova *et al*, 2011; Ikegame *et al*, 2012). We did not use surface markers, another commonly used approach to study cancer stem cells, because the surface marker phenotype for myeloma stem cells remains controversial (Yaccoby & Epstein, 1999; Matsui *et al*, 2008; Jakubikova *et al*, 2011). Similar to the previous study (Jakubikova *et al*, 2011), we demonstrated an identical *IGH* gene rearrangement pattern in the SP cells and mature neoplastic plasma cells (Fig 1), confirming that our methodology is adequate to identify the myeloma stem cells from marrow samples of myeloma patients. In contrast, clonal *IGH* rearrangement was absent in the SP and mature plasma cells of controls when using an identical approach, indicating a lack of clonal populations (data not shown). Additionally, we used another function assay, ALDEFLUOR, for identifying and isolating stem cells of a myeloma cell line (RPMI8226) based on the high expression of aldehyde dehydrogenase of stem cells (Matsui *et al*, 2008; Brennan *et al*, 2010). The stem cells isolated using this functional assay showed similarly elevated CTA expression as isolated by SP cells, confirming that the overexpression of CTA is unique character of myeloma stem cells (Figure S2).

Of the CTA genes up-regulated among the SP of MM, *LUZP4*, *MAGEA12*, *MAGEA3*, *MAGEB2*, *MAGEB3*, and *SSX2*, have particular potential for consideration as targets for immunotherapy targeting the myeloma stem cell-like cells. These CTA genes are localized to the X-chromosome (Table II). As shown by previous studies (Dakshinamurthy *et al*, 2008), CTA genes localized to this chromosome are highly immunogenic; in contrast, immunogenicity of CTA localized on autosomal chromosomes has not been proven (Scanlan *et al*, 2004).

It is worth noting that this study included 8 MM patients (Tables SI, SII); however, it remains difficult to link CTA gene expression profile to the different stages of MM. We believe that more MM cases are required to shed the light on this task.

Immunotherapy, targeting myeloma stem cell-like cells expressing CTA, is particularly attractive for MM patients who have achieved complete remission. The majority, if not all, of these patients developed recurrent disease that is resistant to multiple agents. It has been proposed that the myeloma stem cells, which are highly drug-resistant in nature and have self-renewal capability, play a critical role in the recurrence of MM. This immunotherapy approach is likely to eradicate the myeloma stem cells in these patients to ensure long-term remission of these patients. In our previous studies, we have demonstrated the feasibility of this approach and showed that cytotoxic T-cells (CTL) specific for PRAME-derived peptide can target leukaemic and leukaemic-precursor cells expressing PRAME (Quintarelli *et al*, 2011).

Additionally, previous studies have suggested that some CTA genes may function as regulators of cell-cycle progression, apoptosis and transcriptional repression (Scanlan *et al*, 2002; Yawata *et al*, 2010). CTA genes may thus participate in maintaining the stem cell pool

directly or indirectly. In the present study, knock down *LUZP4* expression with siRNA reduced the colony-forming ability of MM cells and sensitized the cells to chemotherapy. This finding will initiate further investigation regarding the function of CTA genes in MM.

In summary, the current study identifies a subset of CTA genes highly expressed by multiple myeloma stem cell-like side population cells using a unique high throughput qRT-PCR assay designed in our laboratory. The resulting data provides the framework for using immunotherapy to target the myeloma stem cells expressing high levels of CTA for the long-term cure of MM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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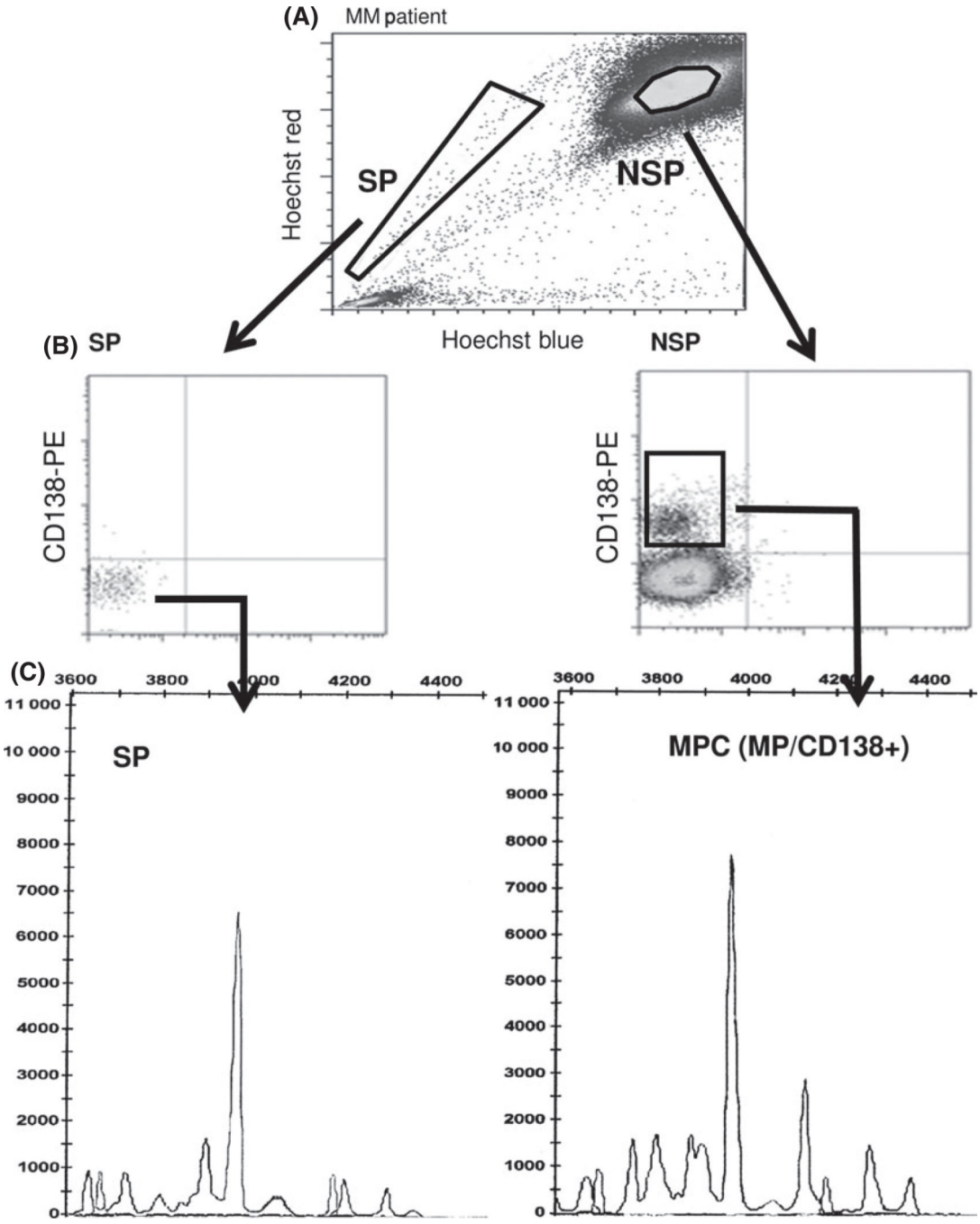

Fig 1.

Side population in primary MM BM sample. (A) Primary MM BM sample was subjected to Hoechst 33 342 SP staining. The SP and non-SP (NSP) are shown. (B) SP and NSP populations were analysed for CD138 expression. (C) *IGH* rearrangement assay was performed in SP and MPC (NSP/CD138+). The experiments were repeated three times, and representative results are shown. MM, multiple myeloma; BM, bone marrow; SP, side population; NSP, non-SP; MPC, mature plasma cells.

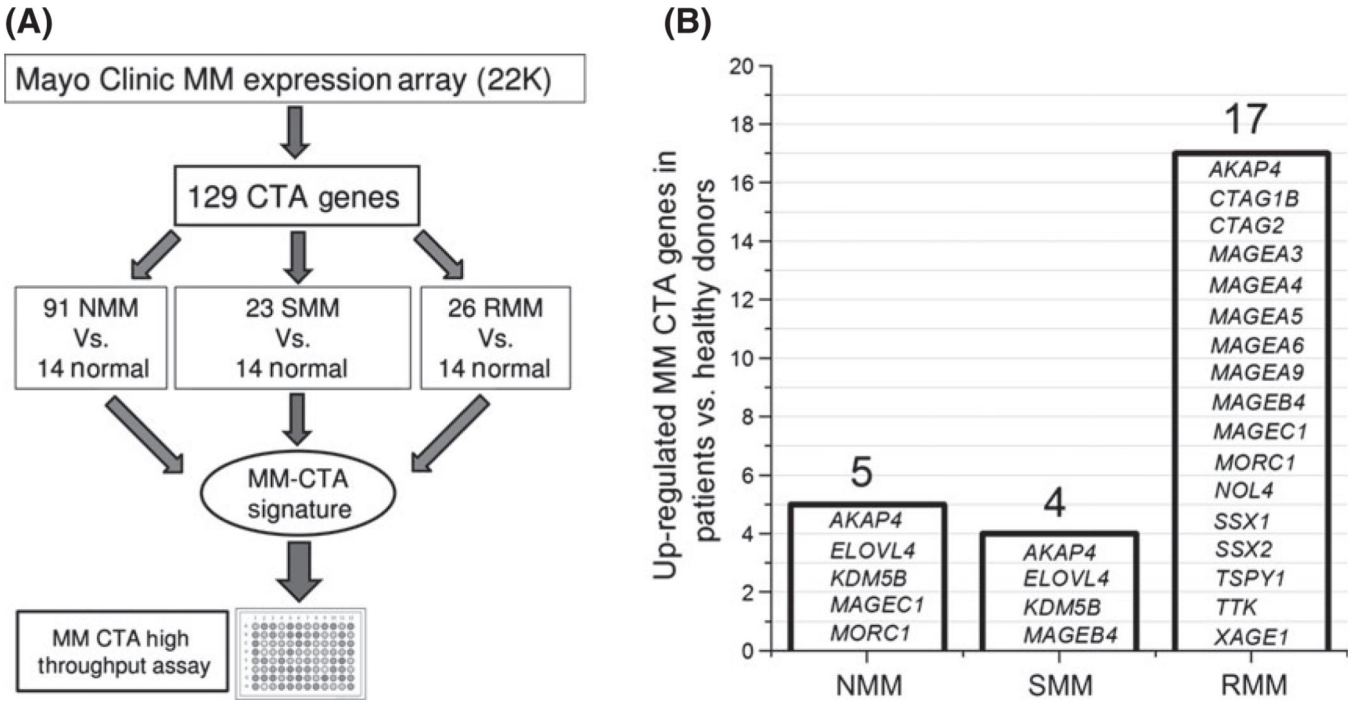

Fig 2.

Design of the MM-specific CTA gene high throughput qRT-PCR Assay. (A) Cancer testis antigen (CTA) gene expression data from Mayo Clinic multiple myeloma (MM) expression array was compared among four groups: new MM patients (NMM), smouldering MM (SMM), relapsed MM (RMM) and normal donors by 1-way ANOVA. If the fold change was >2 (MM: normal) and *P* value < 0·05, the gene was defined as an abnormal up-regulated gene. (B) Highly expressed CTA genes in NMM, SMM, and RMM groups compared with healthy donor are shown.

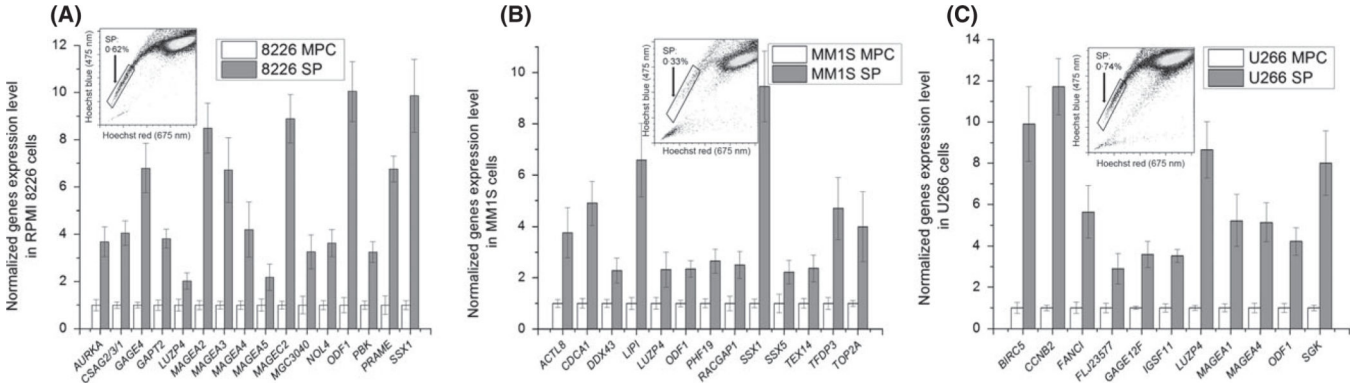

Fig 3.

Up-regulated CTA genes in the SP of MM cell lines compared with MPC. SP and MPC cells were sorted from three MM cell lines and qRT-PCR-based CTA gene assay was carried out. Up-regulated CTA genes in SP (fold change >2 compared with MPC cells, $P < 0{\cdot}05$ after *t*-test) are shown. Data are presented as mean ± standard deviation of three independent experiments after normalizing the data to the MPC. The flow cytometric analysis of SP cells in each cell line is shown as inset. (A) MM cell line RPMI8226. (B) MM cell line MM1S. (C) MM cell line U266. CTA, cancer testis antigen; MM, multiple myeloma; BM, bone marrow; SP, side population; MPC, mature plasma cells.

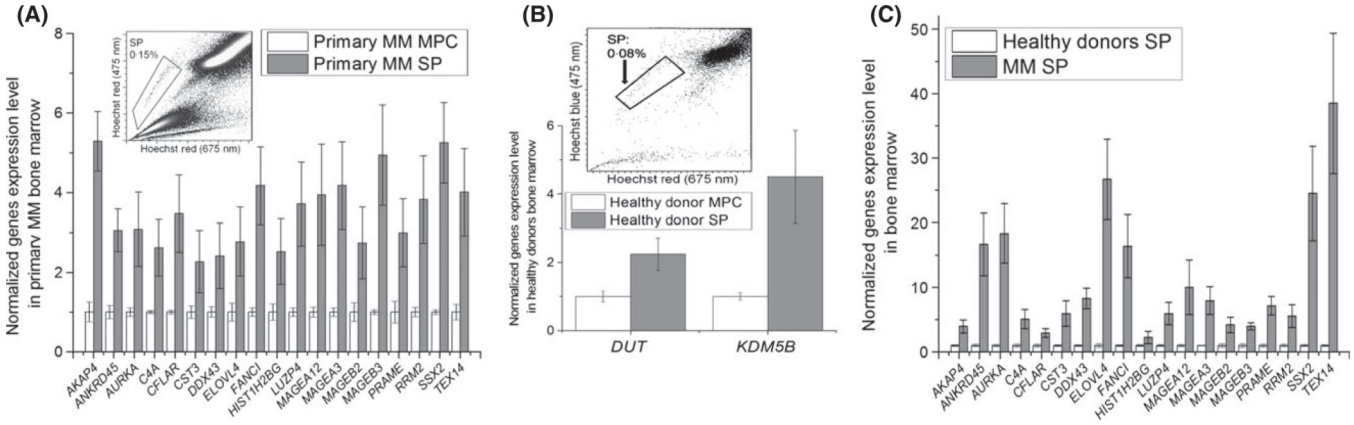

Fig 4.

Up-regulated CTA genes in the SP of primary MM and healthy donor BM compared with MPC. SP and MPC were sorted out from BM of MM and healthy donors and qRT-PCR based CTA gene assay was carried out. (A) Up-regulated CTA genes in 8 MM patient SP cells are shown (fold change >2 compared with MPC, $P < 0{\cdot}05$ after *t*-test). Data are presented as mean of eight MM samples (each has triplicates) ± standard deviation (SD) after normalizing the data to the MPC. The flow cytometric analysis of SP cells is shown as inset. (B) Up-regulated CTA genes in four healthy donor SP cells are shown (fold change >2 compared with MPC, $P < 0{\cdot}05$ after *t*-test). Data are presented as mean of four control samples (each has triplicates) ± SD after normalizing the data to the MPC. The flow cytometric analysis of SP cells is shown as inset. (C) The up-regulated CTA genes in SP of primary MM BM have much lower expression level in SP of healthy donor BM. CTA, cancer testis antigen; MM, multiple myeloma; BM, bone marrow; SP, side population; MPC, mature plasma cells.

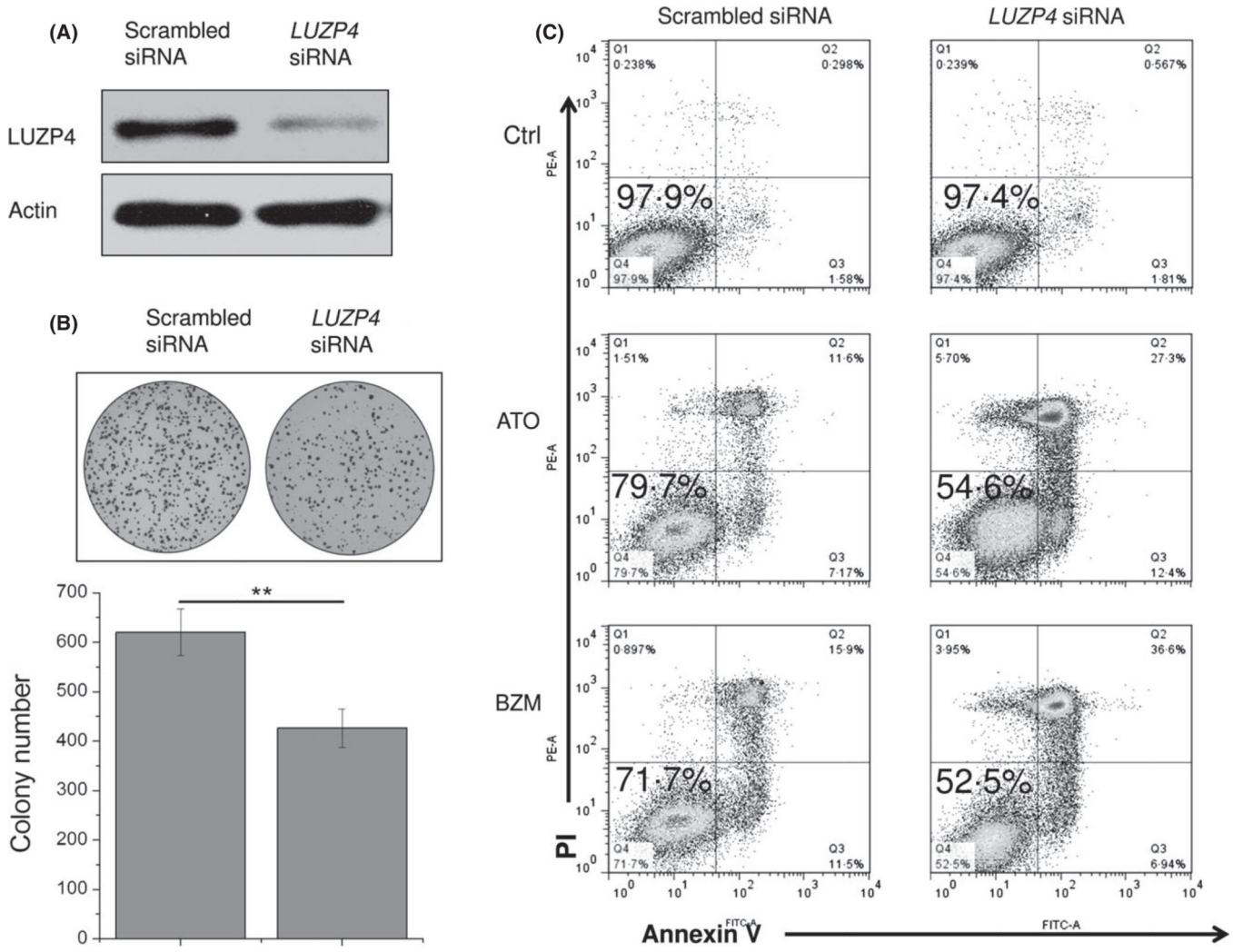

Fig 5.

LUZP4 is related with the colony-forming ability and drug resistance in MM cells. (A) *LUZP4* expression in MM cell line U266 was knocked down with siRNA and the efficiency was verified with Western blot. The experiments were repeated three times, and representative figures are shown. (B) Colony formation assay. 5 × 103 U266 knock down with *LUZP4* siRNA or scrambled siRNA were resuspended in agar and seeded in a 6-well plate. Complete medium was added on the top and changed twice a week. After 2 weeks, dishes were stained with methylene blue, pictures were taken under a phase contrast microscope, and colony number was counted. Representative images are shown from independent experiments (upper panel), and data are presented as mean ± standard deviation of triplicate experiments (***P* < 0·01, *t*-test) (lower panel). (C) Apoptosis assay. U266 knock down with *LUZP4* siRNA or scrambled siRNA were seeded in 6-well plate with complete medium. Cells were treated with 40 nmol/l bortezomib (BZM) or 2 μmol/l arsenic trioxide (ATO) for 24 h followed with flow cytometric apoptosis assay. The percentage of Annexin V/PI double-negative cells was noted. Experiments were repeated three times, and representative blots are shown.

Table I

Up-regulated cancer testis antigen (CTA) genes in relapsed multiple myeloma (MM) patients and their expression level in normal samples, new MM, smouldering MM and relapsed MM. The relative expression level compared with normal and the p value of one-way ANOVA test among different groups is also listed.

Gene symbol	Average of Normal	Average of RMM	RMM/Normal	*P* value	Average of SMM	SMM/Normal	*P* Value	Average of NMM	NMM/Normal	*P* Value
TSPY1	1·31	144·17	109·72	0·0153	2·7917	2·12	0·9802	4·59	3·50	0·9482
CTAG2	1·00	37·10	37·10	0·0085	2·5458	2·55	0·9114	8·42	8·42	0·5272
CTAG1B	1·42	28·10	19·85	0·0071	2·7200	1·92	0·8962	7·24	5·11	0·4917
MAGEA6	10·93	130·44	11·94	0·0013	21·6263	1·98	0·7747	64·96	5·94	0·0890
SSX2	1·01	8·55	8·45	0·0189	1·2571	1·24	0·1563	2·11	2·08	0·6910
XAGE1	7·58	36·04	4·76	0·0019	11·9171	1·57	0·6385	20·91	2·76	0·0893
MORC1	3·91	17·00	4·35	0·0029	7·5021	1·92	0·4167	11·60	2·97	0·0411
MAGEA3	48·13	185·84	3·86	0·0019	52·0454	1·08	0·9301	107·98	2·24	0·1143
SSX1	16·89	50·74	3·00	0·0040	16·5313	0·98	0·9699	25·28	1·50	0·2981
MAGEA5	3·31	8·73	2·64	0·0010	4·1242	1·25	0·621	4·67	1·41	0·3306
NOL4	37·95	96·46	2·54	0·0331	76·7538	2·02	0·1651	81·43	2·15	0·0666
AKAP4	13·86	33·80	2·44	0·0026	44·8713	3·24	≤0·0001	34·75	2·51	0·0003
MAGEA4	14·03	33·62	2·40	0·0110	16·2088	1·16	0·7799	18·81	1·34	0·4689
MAGEA9	27·88	61·35	2·20	≤0·0001	37·2521	1·34	0·1752	45·94	1·65	0·0023
MAGEC1	46·81	101·80	2·17	0·0392	95·0188	2·03	0·0764	112·94	2·41	0·0044
TTK	9·41	20·15	2·14	0·0177	11·1225	1·18	0·7063	13·38	1·42	0·3062
MAGEB4	6·06	12·43	2·05	0·0245	14·0083	2·31	0·0062	8·76	1·45	0·2672

RMM, relapsed multiple myeloma patients; SMM, smouldering multiple myeloma patients; NMM, new multiple myeloma patients.

Table II

Up-regulated cancer testis antigen (CTA) genes in bone marrow from primary multiple myeloma patients. The up-regulated CTA genes in the side population of each multiple myeloma patient, as compared with mature plasma cells, is indicated by "↑".

Gene		**Multiple myeloma cases**							
Symbol	Location	1	2	3	4	5	6	7	8
AKAP4	Xp11.2	↑	↑	↑	↑	↑	↑	↑	↑
ANKRD45	1q25.1	↑	↑	↑		↑	↑	↑	↑
AURKA	20q13	↑			↑	↑	↑	↑	
C4A	6p21.3	↑	↑	↑	↑				↑
CFLAR	2q33–q34	↑	↑		↑	↑	↑		↑
CST3	20p11.21	↑	↑				↑		↑
DDX43	6q12–q13	↑	↑	↑	↑		↑		↑
ELOVL4	6q14		↑	↑	↑			↑	↑
FANCI	15q26.1	↑	↑	↑	↑		↑	↑	↑
HIST1H2BG	6p21.3	↑		↑		↑	↑		↑
LUZP4	Xq23	↑	↑	↑	↑	↑	↑		↑
MAGEA12	Xq28	↑	↑	↑	↑		↑	↑	↑
MAGEA3	Xq28	↑	↑	↑	↑	↑	↑	↑	↑
MAGEB2	Xp21.3	↑	↑	↑	↑	↑			↑
MAGEB3	Xp21.3	↑	↑	↑	↑	↑		↑	↑
PRAME	22q11.22	↑	↑		↑	↑	↑		↑
RRM2	2p25–p24	↑	↑	↑	↑		↑	↑	↑
SSX2	Xp11.22	↑	↑	↑	↑	↑	↑	↑	↑
TEX14	17q22	↑	↑		↑	↑	↑	↑	↑