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The Altered Expression of MiR-221/-222 and MiR-23b/-27b Is Associated With the Development of Human Castration Resistant Prostate Cancer

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Abstract

BACKGROUND—We have previously identified seven miRs—*miR-221*, *-222*, *-23b*, *-27b*, *-15a*, *-16-1*, and *-203*, that are differentially expressed in the hormone sensitive LNCaP cell line and the hormone resistant LNCaP-abl cell line and hypothesized that these miRs may characterize certain subtypes of human castration resistant prostate cancer (CRPC).

METHODS—Functional studies in cell culture systems have been performed to determine the effect of alternated expression level on cellular response to androgen treatment. To determine the clinical relevance of the expression patterns of these miRs, we compared the expression levels of these seven miRs in normal prostate tissues from 86 individuals, prostate tumor tissues from 34 individuals with localized hormone naïve disease, and bone-derived metastatic CRPC tissues from 17 individuals.

RESULTS—The altered expression of *miR-221/-222* (as previously described) or *miR-203* affected the cellular response to androgen treatment, suggesting their potential involvement in the transition to CRPC. However, the expression of *miR-23b*, *-27b*, *-15a*, and *-16-1* did not have a significant influence in the cellular response to androgen treatment, suggesting that these miRs may not play a causative role in the CRPC phenotype. Comparison of the expression levels of these miRs in tissue samples revealed that strikingly, ~90% of the analyzed metastatic CRPC tumors could be characterized by the increased *miR-221/-222* expression and the down-regulated *miR-23b/-27b* expression.

CONCLUSIONS—This finding suggests that altered *miR-221/-222* and *miR-23b/-27b* expression may be associated with the CRPC process.

Keywords

microRNA; miR-221/-222; prostate cancer

INTRODUCTION

Prostate cancer (CaP) is the most common non-cutaneous malignancy diagnosed in men [1]. Androgen and androgen receptor (AR) play crucial roles in CaP development and progression [2]. The development and homeostasis of prostate epithelial cells requires androgen, even in the transformed state. Initially, almost all CaPs are dependent on androgen for growth. Androgen deprivation therapy (ADT), which suppresses testicular androgen production and blocks AR activation, is the most effective therapy for patients with hormone-sensitive CaP (HSPC) [3]. However, most patients eventually become resistant to ADT and progress to castration resistant CaP (CRPC). CRPC proliferates despite castrate levels of serum testosterone and is the lethal form of the CaP. Many attempts have been made to understand mechanisms involved in androgen-independent (AI) growth. It is known that during CRPC progression, CaP cells utilize a variety of AR-dependent and -independent pathways to survive and flourish in an androgen-depleted environment [4–8]. These mechanisms include local production of androgens, a ligand independent but AR-dependent mechanism and AR-independent mechanisms. However, a thorough understanding of the drivers or modulators involved in mechanisms leading to various types of CRPC is still lacking. We postulate that multiple mechanisms may be involved in the development of CRPC in vivo. Thus, a comprehensive study of various pathways that lead to the development and maintenance of CRPCs is a prerequisite for developing effective therapies for this lethal phenotype.

MicroRNAs (miRs), which are groups of naturally occurring single-stranded small non-coding RNAs of 19–25 nt in length, bind with imperfect complementarities to the 3' untranslated region of their target mRNAs to reduce the stability and/or translation efficiency of target mRNAs, in a sequence specific manner [9,10]. It was estimated that the human genome encodes close to one thousand different miRs which are predicted to control the activity of ~30% of all protein-coding genes. Thus, miRs and components of the miR machinery are involved in the control of many cellular functions such as cell proliferation, cell differentiation, stress response, apoptosis, immunity, and transcriptional regulation. Altered expression levels of miRs or dysfunction of miR pathways affect many cellular processes, leading to human diseases, including cancer. Recent studies have documented widespread dysregulation of miRs in virtually all examined tumor types [11–13]. Approximately 50% of miR genes are located in cancer-associated genomic regions or in fragile sites [14]. It is now clear that specific miRs function as critical effectors of several oncogenic and tumor suppressor signaling pathways [15]. MiR expression signatures have been used to classify cancers and to define prognosis [16,17]. Thus, functionally dissecting miR target networks and establishing cancer mechanisms driven by the gain or loss of miR function are crucial for our understanding of cancer development as well as for development of effective clinical applications.

We are interested in molecular mechanisms or modulators involved in the transition of HSPC to CRPC. Increasing evidence has implicated the association of specific miR expression patterns with tumorigenesis and the degree of tumor differentiation in human CaP [18–22]. Nevertheless, the precise role of most identified miRNAs in CaP development and progression still remains to be characterized. We have been focusing on miR signatures in the development and/or maintenance of CRPC. By undertaking a comprehensive miR expression profiling analysis in the androgen-dependent (AD) LNCaP cell line and the LNCaP-derived AI LNCaP-abl cell line, we previously identified seven miRs that were differentially expressed in LNCaP and LNCaP-abl [22]. These seven miRs consist of *miR-203* and three pairs of clustered miRs—*miR-221* and *-222*, *miR-15a* and *-16-1*, and *miR-23b* and *-27b*. The expression of four miRs (*miR-221*, *-222*, *-15a*, and *-16-1*) was significantly up-regulated in LNCaP-abl, ranging from 1.5- to 10.0-fold change, while the

expression of *miR-203*, *-23b*, and *-27b* was reproducibly down-regulated in LNCaP-abl by 4.3-, 1.6-, and 1.9-fold, respectively, compared to those in LNCaP. Among these differentially expressed miRs, the expression of *miR-221* and *-222* were the most dramatically up-regulated in LNCaP-abl, compared to those in LNCaP. Further functional studies in CaP cell lines provided direct evidence implicating the involvement of *miR-221/-222* in CRPC development and/or maintenance [22].

In this article, we study the correlation of CRPC with the above seven cell culture derived-androgen independency associated miRs in human prostate tissues. Since multiple independent mechanisms may mediate the initiation of CRPC, it is possible that different CRPC miR signatures may characterize distinct subtypes of CRPC in patients [4–8]. We compared the expression levels of the seven miRs in human normal prostate tissues, localized HSPC primary tumors (HSPC) and bone metastatic CRPC tumors. Surprisingly, ~90% of analyzed CRPC tumors can be characterized by up-regulation of *miR-221/-222* and down-regulation of *miR-23b* and *miR-27b*. This observation leads to speculation on the potential clinical applications of miRs in CaP.

MATERIALS AND METHODS

Reagents

Synthetic, chemically modified short single stranded RNA oligonucleotides: Pre-miR-molecules, the Pre-miR-negative control, anti-miR molecules, and the anti-miR-negative control were purchased from Ambion. Dihydrotestosterone (DHT) was purchased from Sigma–Aldrich.

Cell Lines

The LNCaP cell line was obtained from the American Type Culture Collection. The LNCaP-abl cell line was provided by Zoran Culig (Innsbruck Medical University, Austria) [23]. LNCaP was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and LNCaP-abl was maintained in RPMI-1640 supplemented with 10% charcoal-stripped FBS.

Transfection

LNCaP and LNCaP-abl cells were cultured to 60% confluence and then transfected with 30 nM of Pre-miR oligonucleotides or anti-miR inhibitors or miR-Precursor negative control and anti-miR inhibitors negative control for 24 hr using Lipofectamine²⁰⁰⁰ according to manufacturer's instructions. Then, cells were treated with ethanol vehicle or 10 nM DHT for another 24 hr.

RNA Extraction and Real-Time RT-PCR

Total RNAs were isolated using *mirVana*TM miRNA Isolation Kit (Ambion). MiR expression levels were quantitated by real-time PCR, using *mirVana*TM qRT-PCR miRNA Detection Kit and Primer Sets (Ambion), following the manufacture instruction. PSA, AR, and GAPDH real-time PCR primer and probes set were inventoried products of Applied Bio-system (Assay ID: Hs01105076_m1, Hs00907244_m1, and Hs02758991_g1, respectively). Analyses were performed using a standard TaqMan PCR Kit protocol. Experiments were carried out in triplicate for each data point.

Cell Proliferation Assay

For all cell growth studies, cells were plated in 96-well plates in androgen-depleted medium. After cultured for 24 hr, cells were transfected with Pre-miRs for 24 hr before adding 10 nM

DHT. Tetrazolium salt (WST-1, Roche Applied Science) cell proliferation assay was then carried out at various days after transfection, following the manufacture instruction.

Prostate Tissue or Tumor-Derived RNAs

Total RNAs from primary CaP tumor tissues and their adjacent normal tissues were as previously described [24]. Briefly, tissue materials were isolated from frozen radical prostatectomy specimen in hormone-naïve patients at Dana-Farber Cancer Institute (DFCI) and Brigham and Women's Hospital and were reviewed by a pathologist. Areas of tumors were selected where >60% of cells consisted of tumor cells. Areas of benign tissue were selected where >50% of cells consisted of non-neoplastic prostate epithelium and were at least 5 mm away from any area of tumor focus. Total RNAs extracted from bone marrow biopsies were as described previously [25]. The frozen bone marrow biopsy sections from patients with CRPC have been carefully examined microscopically and were largely replaced by tumor and minimal residual normal bone marrow elements. High level of PSA amplification, confirmed the presence of CRPC in the samples (data not shown). Total RNAs were extracted using Trizol reagent.

Statistical Analysis

Student's *t*-test and one-way analysis of variance was used to determine the significance. All error bars represent the standard derivation of the mean. Statistical significance for all tests, assessed by calculating two-side *P*-value, was <0.05.

RESULTS

Functional Evaluation of the Role of Putative CRPC-Related miRsin Androgen Dependency of CaP Cell Lines

We chose to use the AD LNCaP and the AI LNCaP-abl cell lines as a model system for identification and functional studies of miRs involved in CRPC development, because the relationship of these two cell lines resemble the progression of CaP from HSPC to CRPC. Additionally, in these two cell lines, the whole genome AR binding sites have been mapped and the dynamics of AR transcription complex loading on PSA regulatory regions are well characterized [26–28]. Thus, we used the expression of PSA; a pharmacodynamic marker of AR activity, to evaluate AR-mediated transcription in response to the DHT treatment in our study.

To address the functional importance of the cell line-derived putative CRPC-related miRs in CRPC development; we determined to what extent the altered expression of each miR affects cell growth and the level of AR-mediated PSA transcription in response to DHT treatment, in LNCaP or LNCaP-abl. To over-express miRs, cell lines were transiently transfected with commercially synthesized miR precursors and to knock down the expression of miRs, cell lines were transiently transfected with commercially synthesized anti-miR inhibitors. With these approaches, we reproducibly found that changing the expression levels of *miR-15a*, *-16-1*, *-23b*, and *-27b* did not significantly affect PSA expression in response to DHT treatment in both LNCaP and LNCaP-abl (Fig. S1-Supplementary Data). This result suggested that these four miRs may not be directly involved in the development of AI phenotype and their differential expression may represent results from the CRPC phenotype.

Changing the expression level of *miR-203* significantly affected PSA transcription in response to the DHT treatment in LNCaP-abl, suggesting its potential involvement in CRPC development. The expression of *miR-203* was decreased in LNCaP-abl by 4.3-fold (*P*=0.004), compared to that in LNCaP, as previously described [22]. Transient over-expression

of *miR-203* in LNCaP-abl significantly increased the level of the DHT induced up-regulation of PSA expression by 1.5-fold in LNCaP-abl cells (Fig. 1A). The over-expression of *miR-203* in transfected LNCaP-abl was verified by RT-PCR (Fig. 1A-left). Transfection with a non-specific miR (Neg RNA) did not affect PSA transcription in response to DHT (Fig. 1A-right); and the AR expression level in LNCaP-abl cells was not significantly affected by over-expression of *miR-203* (Fig. 1A-middle). However, knocking down the expression level of *miR-203* in the LNCaP cell line did not significantly affect PSA transcription in response to the DHT induction (Fig. S1-Supplementary Data).

To corroborate the hypothesis that *miR-203* may play a role in the development of the AI phenotype in LNCaP-abl, we further determined the impact of *miR-203* expression on the growth efficiency of LNCaP and LNCaP-abl, in the presence or absence of androgen. Cell proliferation was measured using the WST-1 assay. The growth efficiency of LNCaP with or without DHT treatment was not affected by transient down-regulation of *miR-203* (data not shown). However, over-expression of *miR-203* in LNCaP-abl reduced the cell growth efficiency in androgen free medium by ~24% while it increased DHT induced cell growth by 1.4-fold (Fig. 1B). Transfection of cells with non-specific Pre-miR did not significantly affect cell growth in the absence or presence of DHT, indicating that simply introducing small RNAs is not sufficient to affect cell growth or growth response to DHT. In sum, our results demonstrated that over-expression of *miR-203* increased the sensitivity of LNCaP-abl to the DHT treatment, suggesting that a low expression level of *miR-203* may be a requisite for maintaining the CRPC phenotype in LNCaP-abl.

Taken together with our previous study, we identified seven miRs that were differentially expressed between LNCaP and LNCaP-abl in total; four of them (*miR-15a*, *-16-1*, *-23b*, and *-27b*) did not exhibit a direct association with the development of androgen independence; and three of them (*miR-221/-222* and *miR-203*) exhibited direct influence on the sensitivity to androgen in CaP cell cultures.

Comparison of miR Expression Patterns in Human Prostate Cancer Tissues

Due to the heterogeneous nature of human CaP cells, we postulated that the cell line-derived CRPC miR signatures might possibly only characterize a subgroup of CRPC in patients. To determine this possibility, we further analyzed the expression levels of the 7 putative CRPC related miRs in 86 normal prostate tissues, 34 localized hormone sensitive primary tumors, and 17 bone metastatic CRPC samples (Fig. 2). As a control, we also measured the 7-miR expression in 10 normal bone marrow specimens. The expression level of each miR was measured by qPCR and normalized with the expression level of U6 RNA.

It appeared that the expression of both *miR-221* and *-222*, which are clustered on the short arm of chromosome X, was significantly up-regulated in metastatic CRPC tumors, by 4.5- and 6.2-fold, respectively (both $P < 0.001$), compared to those of HSPC tumors and by 1.5- and 2.2-fold, respectively (both $P < 0.05$), compared to those of normal prostate tissues (Fig. 2A). The expression levels of *miR-221* and *-222* in metastatic prostate tumors are 2.4-fold ($P = 0.019$) and 4.6-fold ($P = 0.001$), respectively, higher than that in normal bone marrows. The expression of *miR-221/-222* in normal bone marrows is slightly lower than that in normal prostate tissues (see the inset). The elevated expression of *miR-221/-222* in CRPC tumors is consistent with the trend found between LNCaP and LNCaP-abl [22]. However, the expression of *miR-221/-222* was significantly down-regulated in HSPC primary tumors by 3.1- or 2.8-fold (both $P < 0.001$, one-way ANOVA), compared to those in normal prostate tissues (Fig. 2A).

MiR-15a and *-16-1*, sharing the same seed sequence, form a cluster at the chromosomal region 13q14 that is frequently deleted in cancer [21]. It has been documented that *miR-15a*

and *miR-16-1* are significantly down-regulated in prostate primary tumors and can act as putative tumor suppressors by targeting the oncogenes BCL2, CCND1, and WNT3A [21]. Similar to reported data [21], we found that the expression of *miR-15a/-16-1* was significantly down-regulated in prostate primary tumors compared to those in normal tissues by 1.3-fold ($P < 0.05$; Fig. 2B). However, the expression of *miR-15a* was further down-regulated in bone metastatic tumors by 2.4-fold ($P < 0.001$), compared to those in primary tumors, which is contrary to what was observed between LNCaP and LNCaP-abl (LNCaP-abl has an increased expression of *miR-15a*). The *miR-15a* expression level is 1.8-fold lower in bone metastatic prostate tumors than in normal bone marrows ($P = 0.056$). The overall expression level of *miR-16-1* did appear to be higher in CRPC metastatic tumors than in primary tumors, though the difference was not statistically significant ($P = 0.068$, Fig. 2B). There is no significant difference of *miR-16-1* expression level between metastatic prostate tumors and normal bone marrows ($P = 0.165$). We did not observe significant differences of *miR-15a* and *-16-1* expression levels between normal prostate tissues and normal bone marrow tissues (see the inset).

MiR-23b and *-27b* are clustered on Chromosome 9. We found that their expression was significantly decreased in primary tumors compared to those in normal tissues and further decreased in metastatic tumors (Fig. 2C all P -values < 0.001). Compared to the expression level in normal prostate tissues, *miR-23b* or *-27b* was significantly down-regulated in the primary prostate tumors by 1.5- or 1.8-fold, respectively. The *miR-23b* and *-27b* expression levels were further decreased in metastatic CRPC samples, by 2.8- or 3.1-fold, respectively, compared to primary prostate tumors. The expression levels of *miR-23b* and *-27b* are 3.2-fold ($P = 0.009$) and 2.1-fold ($P = 0.047$) lower in metastatic prostate tumors than in normal bone marrows. The expression of *miR-23b* and *-27b* in normal bone marrows is slightly lower than that in normal prostate tissue, but this was not statistically significant (see the inset). The down-regulation of *miR-23b/-27b* in CRPC tumors consistent with that observed between LNCaP and LNCaP-abl (a decreased expression of *miR-23b/-27b* in LNCaP-abl).

The expression level of *miR-203*, was not differentially expressed between prostate normal tissues and primary tumors over all (Fig. 2D). However, metastatic tumors exhibited a higher expression level of *miR-203*, compared to those in primary tumors; this event is contrary to what was observed between LNCaP and LNCaP-abl. There is no significant difference in *miR-203* expression level between metastatic CRPC prostate tumors and in normal bone marrows ($P = 0.85$). The expression of *miR-203* in normal bone marrows is slightly higher than that in normal prostate tissues (see the inset).

In summary, in line with our previous findings in LNCaP and LNCaP-abl, expression of *miR-221/-222* was markedly increased and expression of *miR-23b/-27b* was significantly decreased in metastatic CRPC tumors, compared to those in HSPC tumors. The expression patterns of *miR-15a*, *miR-16-1*, and *miR-203*, which were observed in cell lines, are not consistent with those observed in human prostate tumors tissues. We hypothesized that our previously observed differential expression patterns of *miR-15a/-16-1* and *miR-203* may be unique to the transition of LNCaP to LNCaP-abl and the differential expression patterns of *miR-221/-222* and *miR-23b/-27b* may potentially characterize the transition of HSPC to CRPC.

Representation of the Differential Expression Patterns of *miR-221/-222* and *miR-23b/-27b* in Bone Metastatic CRPC Tumors

Since the potential diversity of the CRPC characters exists among patients we further compared the expression patterns of *miR-221/-222* and *miR-23b/-27b* in each individual bone metastatic prostate tumor samples, in relative to their corresponding mean expression level in primary tumors, to determine the abundance of patients who carry LNCaP-abl like

CRPC among the 17 metastatic CRPC tumor samples (Fig. 3). Surprisingly, expression of *miR-221/-222* is up-regulated in 16 out of 17 CRPC tumors ($P < 0.001$) and 15 out of 17 CRPC tumor samples exhibited down-regulated *miR-23b* and *miR-27b* ($P < 0.001$), compared with their mean expression value of primary tumor samples. Fourteen out of 17 CRPC samples had both the up-regulation of *miR-221/-222* and the down-regulation of *miR-23b/-27b* (Fig. 3), similar to the event observed in LNCaP-abl compared to that in LNCaP.

Down-Regulation of 6 miRs Expression in Prostate Primary Tumors

As demonstrated in Figure 2, showing the comparison of overall expression of the seven putative CRPC-related miRs in prostate tumors, the expression level of *miR-221*, *-222*, *-15a*, *-16-1*, *-23b*, and *-27b* were significantly decreased in primary tumors, compared to normal tissues. The lowered expression of these miRs in malignant prostate tissues was further confirmed when their expression was compared in 21-paired primary prostate tumors and their adjacent normal prostate tissue (Fig. 4). We found that 19 of 21 tumors showed down-regulation of *miR-221*, *-222*, and *-23b* in primary tumors compared to their normal counterparts (all three P -values < 0.001 , paired t -test). *miR-27b*, *miR-15a*, and *miR-16-1* were also down-regulated in ~80% of tumor samples ($P = 0.001$, 0.015 , and 0.008 , respectively). The expression level of *miR-203* was not significantly altered in primary tumor tissues, compared to their normal counterparts ($P = 0.121$). This result suggested that down-regulation of *miR-221*, *-222*, *-23b*, *-27b*, *-15a*, and *-16-1* may accompany the process of prostate tumorigenesis. This observation is consistent with miR profiling analysis previously reported by Tong et al. [29], Schaefer et al. [30], and Bonci et al. [21].

DISCUSSION AND CONCLUSION

Extensive evidence has indicated that miRs play an important role in controlling many fundamental cellular processes and in the pathogenesis of cancer [10–13]. A limited number of studies have investigated the role of miRs in CaP initiation and progression [19–21]. Extensive dysregulation of miRs was consistently found in CaP [18–21]. It is possible to distinguish carcinoma from non-neoplastic cells or subgrouping tumors based on their androgen dependence by miR expression profiles [18]. We have been interested in mechanisms involved in the transition of CaP to CRPC and the development of better predictive factors for CRPC. To achieve our goals, we initiated the study of miR expression signatures of CRPC. Our previous miR expression profiling identified seven miRs, *miR-221*, *-222*, *-15a*, *-16-1*, *-203*, *-23b*, and *-27b*, that are differentially expressed in the AD, LNCaP and the CRPC, LNCaP-abl cell lines. Further functional validation in cell culture systems demonstrated that the expression level of *miR-221/-222* (as previously shown, 22) or *miR-203* significantly affected the sensitivity of cells responding to treatment with DHT as assayed by their effects on the PSA transcription and cell growth, while the expression level of *miR-15a*, *-16-1*, *-23b*, and *-27b* did not affect the sensitivity of LNCaP and LNCaP-abl responding to the treatment of DHT. This result suggested that *miR-221/-222* and/or *miR-203* may be directly involved in essential functions in the progression of LNCaP to CRPC LNCaP-abl and that *miR-15a*, *-16-1*, *-23b*, and *-27b* may not be directly involved in the development of androgen independency in LNCaP-abl and their differential expression may be secondary to LNCaP progressing to LNCaP-abl. The impact of *miR-203* expression level on the sensitivity to the DHT treatment was only observed in the LNCaP-abl cell but not in LNCaP. Currently, we do not understand the exact mechanistic reason for this differential effect. It is possible that down-regulation of *miR-203* expression alone is not sufficient for the initiation of CRPC, though it may be required for the maintenance of CRPC in LNCaP-abl.

To establish the clinical relevance of these cell line-derived putative CRPC miR-signatures, we compared the expression levels of these seven miRs in normal prostate tissues, HSPC primary tumors and the CRPC bone metastasis tumors. It appeared that the differential expression patterns of *miR-15a*, *-16-1*, and *-203*, as observed in LNCaP and LNCaP-abl cannot be identified in the human CaP specimens. We hypothesized that the differential expression of *miR-15a*, *-16-1*, and *-203* may result from the transition of LNCaP to LNCaP-abl. However, up-regulation of *miR-221/-222* and down-regulation of *miR-23b/-27b* were observed in association with the human CaP progressing to CRPC. More than 90% of CRPC samples examined had both up-regulation of *miR-221/-222* and down-regulation of *miR-23b/-27b*, compared to HSPC primary tumors. *miR-221* and *-222* appear to be directly involved in the development of androgen independency, as previously described [22], while *miR-23b* and *-27b* do not have a direct involvement in the initiation of the AI phenotype. Porkka et al. [18] previously reported the association of down-regulation of *miR-221/-222* with CRPC however, their observation was found in the transurethral localized tumors of CRPC patients. Spahn et al. [31] reported that miR-221 was found to be reduced in more aggressive prostate cancers and to be associated with recurrence. Though, all samples (paired primary tumors and lymph node metastasis) used in their study were taken from patients who have never received neoadjuvant hormonal-, radiation-, or chemotherapy (i.e., patients of hormone naïve disease). Thus, Spahn et al.'s report did not address issues relating to the CRPC cell. Consistent with the study by Spahn et al., we also observed the reduced expression of miR-221 in tumors from patients of localized hormone naïve disease. However, in our analysis, *miR-221/-222* was significantly up-regulated in bone metastatic CRPC cells. Due to the great heterogeneity in CaP pathogenesis, we were surprised by the consistency in the finding of differential expression of *miR-221/-222* and *miR-23b/-27b* in CRPCs.

We have hypothesized that the up-regulation of *miR-221/-222* expression may have influenced essential components of the AR functional integrity, as a result, leading to the development and/or maintenance of the CRPC phenotype. Interestingly, the up-regulation of *miR-221/-222* expression is needed to maintain the TRAIL-resistant phenotype in human non-small cell lung cancer and liver cancer via c-kit, PTEN, and TIMP3 [32,33], and it also is associated with conferring tamoxifen resistance, fulvestrant resistance, and cisplatin resistance in breast cancer [34–37]. The concomitant observation of the correlation of up-regulation of *miR-221/-222* with the reduction of susceptibility to treatment in different types of cancers and therapies is intriguing. We suspect that different or multiple *miR-221/-222* target molecules may be involved in the development of resistance in different critical pathways. Currently, p27/kip1, p57kip2, c-kit, Bim, ER, PTEN, TIMP3, and PUMA have been reported to be *miR-221/-222* targets that are involved in cancer progression or development [33–36,38–44]. We have established in cell model systems that all these known *miR-221/-222* targets do not appear to be involved in the CRPC phenotype of LNCaP-abl. However, the possibility that some of these *miR-221/-222* targets may be involved in the development of CRPC in patients is not excluded. Mechanisms involved in the up-regulation of *miR-221/-222* in LNCaP-abl and the CRPC tumor tissue are not known. We are currently investigating the molecular details of *miR-221/-222* function in the initiation of CRPC and characterizing *miR-221/-222* targets relevant to the transition of LNCaP to the CRPC, LNCaP-abl.

It has been shown that MYC protein transcriptionally represses *miR-23b*, leading to up-regulation of the mitochondria glutaminase and glutamine metabolism, providing an energy resource for cancer cell proliferation [45]. Nuclear over expression of MYC protein occurs frequently in primary and metastatic prostate carcinoma and its expression level appeared significantly higher in metastatic prostate carcinoma than that in primary prostate carcinoma [46]. We hypothesize that the significant down-regulation of *miR-23b* in CaP tissues could

be due to the over-expression of MYC and the enhanced glutamine metabolism may have partly sustained the growth of CRPC cells.

In sum, our study shows consistent changes in the expression of *miR-221/-222* and *miR-23b/-27b* between localized prostate primary tumor cells and CRPCs. This finding suggests a potential role for *miR-221/-222* and *miR-23b/-27b* as markers or predictors for CaP progression to CRPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	androgen dependent
AI	androgen independent
ADT	androgen deprivation therapy
AR	androgen receptor
CaP	prostate cancer
CRPC	castration resistance prostate cancer
miR	microRNA
DHT	dihydrotestosterone

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Fig. 1.

The impact of *miR-203* expression levels on the AR-mediated transcription and the growth of LNCaP-abl. **A:** The influence of *miR-203* expression levels on the AR-mediated transcription. The left panel shows the confirmation of the expression levels of *miR-203* by semi-quantitative RT-PCR in transfected LNCaP-abl cell lines. The expression of U6 was used as a control. The middle panel shows the AR expression in transfected cell lines. The right panel shows the PSA expression level. The expression of AR and PSA was measured by qRT-PCR. Total RNAs were isolated from LNCaP-abl cells that were mock-transfected (–), and transfected with pre-miR-203 (203) or pre-miR-Neg RNA (Neg RNA) and treated with (+) or without DHT (–). In all experiments, the relative expression levels of AR and PSA were normalized by the expression level of GAPDH. Values represent the fold differences relative to those in cells without any drug treatment and transfected with pre-miR-Neg (Neg RNA), which were set as 1.0. *Indicates that the fold changes of those transfected samples compared with their corresponding negative controls show a *P*-value <0.05 in one-way ANOVA. **B:** The impact of *miR-203* on LNCaP-abl growth. WST-1 analysis was used to measure the growth of LNCaP-abl that were transfected with Pre-miR-203, miRNA-precursor-negative control (Neg), mocked transfected (lipo 2000), and kept in medium with (+DHT, solid lines) or without androgen (broken lines). Triplicate experiments were performed for each set. The data represent mean ± SD (N = 3).

Fig. 2.

MiR expression patterns in normal prostate tissues, primary tumors, and CRPC tumors. The expression patterns of *miR-221/-222* (A), *miR-15a/-miR-16-1* (B), *miR-23b/-27b* (C), and *miR-203* (D) were summarized. Expression level of miRs was measured by Taq Man qPCR on 85 normal prostate tissues (white boxes), 34 primary prostate tumors (light gray boxes), 17 metastatic CRPC tissues (dark gray boxes), and 10 normal bone marrow samples. Inserted plots in each panel indicated relative expression levels of according miRs in normal prostate tissues (white bars with P) or normal bone marrow samples (gray bars with B). All expression levels were normalized by the expression level of U6 RNA. Values represent the fold differences relative to the mean expression level of normal tissues, which was set as 1.0. P values were estimated by one-way ANOVA. NS indicates that the P -value is not significant.

Fig. 3.

Expression patterns of CRPC related miRs in each individual CRPC tumor. **A:** The relative expression level of miR-221, -222, -23b, and -27b in each individual bone metastatic CRPC tumor sample, comparing to their corresponding mean expression level in primary tumors (which was arbitrarily set as 1). One to 17 indicate CRPC tumor samples from 17 individual patients, respectively. **B:** Classification of CRPC tumors based on the expression pattern of miR-221, -222, -23b, and -27b. ↑ and ↓ indicate that the expression of miRs is increased or decreased, respectively, in CRPC tumors (or LNCaP-abl) compared with their mean expression value detected in primary tumors (or in LNCaP).

Fig. 4.

MiRs expression in prostate tumors compared with patient-matched controls. The expression of *miR-221*, *-222*, *-15a*, *-16-1*, *-23*, *-27*, and *-203* was analyzed by TaqMan qPCR and normalized with the expression level of U6, which serves as an endogenous control. Relative miRs expression levels were determined in tumors (black bars) and normal tissues (gray bars) relative to the mean value of each relevant miR expression in all normal tissues, which was set as 1.0. The order of tissue samples is the same for all seven plots. The experiment was done in duplicate. The difference of miR expression level between tumors and their matched normal control was indicated by *P* values, estimated by paired *t*-test.