Occult Hepatitis B Virus Infections (Often with Human Herpesvirus 7 Co-Infection) Detected in Pityriasis rosea Patients: A Pilot Study

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Abstract

Background: The etiopathogenesis of Pityriasis rosea (PR), a papulo-squamous skin disease, remains elusive and hypothesized to be caused primarily by human herpesvirus (HHV) 6 or 7 or immune dysfunction. Aims: The recent increasing incidences of hepatitis B virus (HBV) infections, including asymptomatic occult HBV infections (OBIs), in a densely populated city in India, prompted us to investigate whether PR patients (from varied socioeconomic and immune status) harbor the underlying HBV infections. These cases were also investigated for HHV 6 and 7 infections. Materials and Methods: DNA from ethylenediaminetetraacetic acid blood samples from PR-diagnosed individuals (n = 13; mostly young adults) and healthy controls (n = 11) were subjected to virus gene-specific polymerase chain reactions (PCRs) for HBV and HHV 6 and 7. PCR products of expected length, when observed, were sequenced (bidirectional) using overlapping primers. Sequences were identified by NCBI BLAST and analyzed by multiple sequence alignment and phylogenetic studies. The blood samples were tested for HBsAg by EIA. Results: In 5/13 PR samples, only HBV DNA (4/5 being HBsAg negative) was detected, providing first-time evidence that PR may be manifested in asymptomatic HBV carriers. 6/13 cases were HHV 7 (not HHV 6) DNA positive, providing confirmatory molecular genetic evidence for the first time of PR association with HHV 7 from India. Surprisingly, 5/6 HHV 7-positive PR cases were also HBV positive. Overall, 10/13 PR samples showed evidence of HBV infection. 8/13 were OBI, harboring at least one OBI-signature S protein mutation. All healthy controls were HBsAg EIA and PCR negative. Conclusions: 77% of PR patients presented the evidence of underlying HBV infection (genotype D2), suggestive of horizontal HBV transmission. This warrants for mass HBV vaccination. PR patients should be tested for underlying virus infections for appropriate therapy and management.

Key Words: HBsAg, human herpesvirus 7, occult hepatitis B virus infection, polymerase chain reaction, Pityriasis rosea

Introduction

Occurrence of Pityriasis rosea (PR) is ubiquitous; incidence recorded at 0.39–4.80 per 100 dermatology patients¹ and can occur at any age, most often between the ages of 10 and 35 years. PR may pose a risk for spontaneous abortion in pregnant women.² The disease is self-limiting; clinical course in some cases is prolonged (over months) and recurrence is known to occur, thereby posing diagnostic and management challenges.

Hypotheses have been put forward regarding the exact cause of PR, suggesting both infective agents and noninfective etiologies such as autoimmunity and atopy.³ However, many authors are of opinion that the distinct clinical course of PR points toward an infectious etiology. This led to the evaluation of cytomegalovirus, Epstein–Barr virus, parvovirus B19, picornavirus, influenza and parainfluenza viruses, Streptococcus spp., Legionella spp., Mycoplasma spp., and Chlamydia spp. infections; but concrete evidence of their association with PR could not be established.⁴

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How to cite this article: De A, Roy S, Sukla S, Ansari A, Biswas S. Occult hepatitis B virus infections (often with human herpesvirus 7 co-infection) detected in pityriasis rosea patients: A pilot study. Indian J Dermatol 2017;62:598-605.

Received: May, 2017. Accepted: October, 2017.
Accumulating evidences, to date, suggest that PR may be caused by or at least strongly associated with the reactivation of latent human herpesvirus 7 (HHV 7) and/or HHV 6,[5-8] but this concept is also not free from contradictory findings.[4] In the present study, PR samples from a hospital in Kolkata, India, were screened for possible association with HHV 7 and/or HHV 6 infection as there are no confirmatory molecular genetic studies available from the Indian subcontinent linking PR with HHV 7 or 6 infections.

Recent studies have shown that other skin conditions such as psoriasis (PsO) require different management strategies in case of hepatitis B (HBV)- or C virus (HCV)-infected patients.[9] However, globally, the reports of the association between PR and HBV are sparse. In the context of high incidence of HBV cases (including occult HBV) in the recent times reported from Kolkata,[10,11] it was interesting to investigate whether the patients apparently presenting with PR also harbored the underlying chronic HBV infections.

Materials and Methods

Ethics statement
The present study was approved by the respective Institutional Ethical Committee of CSIR-Indian Institute of Chemical Biology and Calcutta National Medical College (CNMC), Kolkata, with written informed consent (in their native language) being obtained from all the patients/individuals before their blood was collected.

Study subjects
Two independent consultant dermatologists had examined the patients and only those patients were chosen for sample collection when both have diagnosed the cases as PR. Typical herald patch, peripheral scaling, and asymptomatic lesions in “Christmas tree” pattern or distribution were the essential constituents of such diagnosis.[12,13]

Approximately 2 ml of ethylenediaminetetraacetic acid blood sample was obtained from each of the 11 healthy and 13 individuals presenting with the symptoms of PR in the Outdoor Clinic, Department of Dermatology, CNMC, during 2016. The blood samples were stored at −80°C in a freezer until further analysis.

DNA extraction and polymerase chain reaction
DNA was extracted from the blood samples using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) or DNeasy Blood and Tissue kit (Qiagen, Germany) following manufacturers’ instructions.

The extracted DNAs were subjected to virus-specific polymerase chain reactions (PCRs) using GoTaq 2X Master Mix (Promega, USA) with nested sets of virus gene-specific primers. For HBV, the first-round PCR to amplify the S gene was performed with SPL3 and SPL2 primers while the nested PCR was performed on 10-fold-diluted first-round products using the second set of internal primers, SPL4 and SPL5, as described previously [Table 1].[14]

The sample DNAs were also tested for HHV 6 and HHV 7 using respective virus-specific nested set PCRs as described previously [Table 1].[15]

All PCRs have been repeated twice for each DNA test sample. PCR products were resolved by 1% agarose gel electrophoresis. PCR bands of correct size were either gel purified (Qiagen Gel Extraction kit, Germany) or PCR purified (Qiagen PCR Purification kit, Germany) prior to DNA sequencing.

Sequence analysis
Nucleotide sequences were confirmed by bidirectional sequencing of the purified PCR products using the same primers used for PCR and for HBV sequences, additionally also by overlapping internal forward and reverse primers [Table 1].

DNA sequences of HHV 7 PCR-positive samples were subjected to NCBI BLAST for confirmation of identity. Sequences

| Table 1: Primers used in polymerase chain reactions and for DNA sequencing |
|---|---|---|
| **Primers** | **Sequence 5’-3’** | **Product size (bp)** |
| HHV 6-OF | GTTTTCATTTGATCAGAAGCCGT | 783 |
| HHV 6-OR | CATTACATCAATGGTCATACAT | |
| HHV 6 FN | CCTGTGTAGGTCGAATGCGAC | 492 |
| HHV 6 RN | AAGCCGAGCAACACATGCCAGGC | |
| HHV 7 OF | ATCCCCAGCTTTGTCATAAGTAC | 186 |
| HHV 7 OR | GCCITTGCAGCTACATGATTTTTG | |
| HHV 7 FN | CGAAATGACGACGATGTTG | 124 |
| HHV 7 RN | TAGATTTTGAAAGAATTTAATAAC | |
| SPL3 | GCCGGCCAGTACCACTGCCTGGGAARCAAYCRATCRGGA | 1652 |
| SPL2 | GCCITTTGCAAAGTCACTACAAATGCTATGTC | 1277 |
| SPL4 | ACCACAGATCAGTACTGAGTYGTG | |
| SPL5 | GCTTCGAAACGACRGCAGRCAGAAC | |
| HBV-2F | GTGCTGGGCTTTGTTATCAC | |
| SB-742R* | CATCAAATGACACAGGCACTGA | |
confirmed by reads from at least one forward and one reverse primer were aligned using ClustalW. The HHV 7 sequences from this study were aligned with \textit{UL10} structural phosphoprotein gene of closely related HHV 7 strains (retrieved from GenBank), while the HBV sequences were aligned with other closely matched HBV genotype D sequences.

Phylogenetic analysis was done using the Neighbor-Joining method using MEGA 7 on 633 nt of HBV S gene (pertaining to nt positions 325–957 as in HBV isolate KC875340-West Bengal-India-genotype D) to determine the genetic distances of the HBV isolates from the PR samples in relation to other closely related HBV strains.\textsuperscript{[16]}

\textbf{Testing Pityriasis rosea blood samples for HBsAg by ELISA}

ELISA was performed (as per manufacturer’s instruction) on the blood samples using the Monolisa HBsAg ULTRA kit (Bio-Rad, France) to detect HBV surface antigen (HBsAg) in human blood. Volume used was 100 µl for each sample. Results were confirmed for each sample by repeating ELISA two more times.

\textbf{Results}

The main findings of this study were as follows:

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Sample number & Sex & Age (years) & HBsAg (ELISA) & PCR \textsuperscript{[17]} & OBI-specific mutation(s) & Other notable mutation(s) \\
\hline
S1 & Male & 7 & - & - & - & - \\
S2 & Male & 13 & - & - & - & - \\
S3 & Male & 4.5 & - & - & + & - \\
S4 & Male & 10 & + & - & + & - \\
S5 & Female & 14 & - & + & + & Q101H, G145A, T118V/A128V \\
S6 & Female & 8 & - & + & + & M133I \\
S7 & Male & 21 & - & + & + & M133I, P178R \\
S8 & Female & 16 & + & + & + & Q101H, G145A \\
S9 & Female & 35 & - & + & - & M133I \\
S10 & Male & 16 & - & + & - & Q101H, G145A \\
S11 & Male & 20 & - & + & - & M133I \\
S12 & Male & 30 & - & + & - & M133I \\
S13 & Male & 12 & + & + & - & M133I \\
\hline
Healthy controls (n=11) & 5 male/6 female & 15 (median) & - & - & - & - \\
\hline
\end{tabular}
\caption{Summary of serological and molecular analyses of blood samples}
\end{table}

NA: Not applicable, PCR: Polymerase chain reaction, HHV: Human herpesvirus, HBV: Hepatitis B virus, HBsAg: HBV surface antigen, OBI: Occult HBV infection, "−" denotes negative and "+" denotes positive result, within the limits of detection of the assay used

Nine of the 13 (69.2%) PR samples were found to be HBV DNA positive [Table 2] by HBV S gene-specific-nested PCR yielding PCR bands of expected length (1277 bp) [Figure 1a]. All these samples were found negative for first-round PCR by HBV S-gene-specific primers (expected band length 1652 bp) [Figure 1a]. One of the remaining samples was HBsAg positive but HBV DNA negative. Overall, 10 out of the 13 PR samples (77%) showed evidence of HBV infection including successful S gene sequence retrieval for nine of them [Figure 2a and Table 2].

Six of the 13 (46%) PR samples were also found positive for HHV 7 by nested PCR [Figure 1b] and sequencing [Figure 2b]. None of the samples were positive for HHV 6-specific nested PCR (data not shown).

Four of the above HHV 7-positive cases were also HBV DNA positive and another HHV 7-positive sample (S4) was HBsAg positive but HBV DNA negative. In all, 5 of the 13 (38%) PR samples showed evidence of HHV 7 and HBV co-infection. Only one sample (S3) showed evidence of HHV 7 infection only and no HBV involvement. The most intriguing finding was that five of the PR samples were exclusively HBV -DNA positive including one being HBsAg positive (S13). Neither HHV 7 nor HHV 6 could
be detected in any of these cases. This is the first report of possible PR association with the underlying asymptomatic HBV infection.

Three PR samples (S4, S8, and S13) were found to be positive for HBsAg by ELISA [Table 2]. The average S/CO values (± SD) for the samples S4, S8, and S13 were 1.2 ± 0.2, 1.1 ± 0.1, and 1.2 ± 0.1, respectively, from three independent experiments done in duplicate. As specified by the kit, S/CO value repeatedly >1.0 confirms HBsAg positivity. The underlying virus infection could not be detected for only two of the 13 PR cases studied (S1 and S2).

Sequence alignment and phylogenetic analysis revealed that the HBV S genes detected in the PR samples were different but all belonged to genotype D2 [Figure 3]. The S protein amino acid sequences could be retrieved partially in four of the 13 PR samples and completely in two of the PR OBI samples (S8 and S13). Blood samples from all healthy controls were HBsAg EIA and PCR negative.

The HBV-positive PR samples were from patients whose age ranged from 8 to 35 years (median age = 16 years) while the HHV 7-positive samples were from patients with age ranging from 5 to 21 years (median age = 12 years).

**Discussion**

It is intriguing to observe that >75% of the patients (10 of 13) presenting with PR in a hospital setting in Kolkata were HBV-DNA or HBsAg positive in blood, suggestive of the underlying HBV infection although they are asymptomatic for such condition. Only three of the PR HBV samples tested positive for HBsAg by ELISA, suggesting that the remaining seven PR HBV cases fit the definition of cryptic or “occult” HBV infections (OBI), i.e., HBsAg negative but HBV DNA positive outside the seroconversion window period.\(^1\)

Although the frequency of OBI varies globally with higher prevalence in the developing countries, some reports from eastern part of India (Kolkata) suggested an alarmingly high prevalence of OBIs in the recent past.\(^6,11\) This corroborates well with our detection of OBI at high frequency among PR patients in Kolkata. In support of this proposition, at least one OBI-associated amino acid mutation has been identified in the S protein sequences that could be retrieved from the HBV DNA-positive PR patients.

The OBI-associated amino acid mutation, sG145A, within the HBsAg Major Hydrophilic Region (MHR) reported to be responsible for failure of S protein detection in enzyme immunoassays (EIAs) as well as vaccine escape, was recorded in two of the PR OBI samples (S5 and S10).\(^18,20\) The same samples also contained sQ101H mutation known to be associated with reduced sensitivity to EIAs. sQ101H has been reported to cause 13-120-fold reduced sensitivity with different HBsAg diagnostic EIA kits.\(^22\) Both mutations had been, however, rarely encountered in HBsAg-positive cases, as has been seen in one of the HBsAg-positive PR samples (S8) in the present study.

Another notable OBI mutation observed in the remaining five PR OBI cases was sM133I (within MHR), also known to be associated with reduced sensitivity to EIAs.\(^22-24\) One of these sM133I-containing PR OBI patients also harbored the sP178R mutation, which is an OBI-defining extra-MHR mutation, responsible for S protein secretion defect from HBV-infected hepatocytes.\(^25\)

All PR HBV DNA-positive samples also harbored P127T mutation and the T118V/A128V double mutation [Figure 2a]. P127T has been reported to be the most frequently occurring S gene mutation in genotype D.\(^26\) In fact, the above trio of mutations in genotype D/ayw3 has been reported to be associated with reduced HBsAg reactivity.\(^27,28\) There is hardly any previous report on the association of HBV infection with PR. One patient was reported with the development of PR post-HBV vaccination.\(^29\) Another case report
described a female patient suffering from chronic HBV infection, who developed atypical PR-like eruptions after administration of the second dose of polyethylene glycol-interferon-alpha 2a.

Previously, a randomized, double-blind, placebo-controlled study was done on 73 PR patients from South India, which demonstrated that high dose of acyclovir was effective in the treatment of PR. Around 53.3%–86.7% of PR patients from the acyclovir-treated groups responded favorably to the herpes antiviral, acyclovir, suggesting that HHV 6 or 7 viral infections were involved in causing PR. We confirm from our study that a considerable fraction of PR cases from India do have association with HHV 7 infection as 46% (n = 6/13) of...
PR cases were HHV 7 DNA positive in the blood (and HHV 6 DNA negative) within the limits of PCR detection. We believe that this is the first report of unequivocal evidence at molecular level of association of PR with HHV 7 reactivation from India.

Five of the six HHV 7-positive PR cases were also concurrently HBsAg (one case) or HBV DNA positive (OBI). None of these patients presented with any symptom typical of hepatitis such as abdominal pain or jaundice and were, therefore, asymptomatic for the underlying HBV infection. Hence, there is a possibility that PR manifestation was due to HHV 7 reactivation, the latter being augmented by altered/lower immunity caused by underlying OBI.

All HBV cases detected are of genotype D2. It is not clear whether this genotype has any specific bearing with PR manifestation or simply because it is the most prevalent genotype circulating in India, including Kolkata. Another possible explanation could be molecular mimicry in which antibodies against a foreign antigen/virus cross-react with self-antigens leading to auto-immune disorders which can also externally manifest in the form of skin disorders such as Pso. Since altered immunity/immunosuppression is common in chronic HBV patients such as OBI, it is a possibility that skin diseases such as PR or Pso are visible manifestation of such altered/reduced immunity. This is supported by the five PR cases which showed the evidence of underlying HBV infection but were negative for HHV 7 or 6. It is, therefore, interesting to note that the underlying chronic/cryptic HBV infection may alone produce skin lesions diagnosed as typical PR in the clinics. In this context, it is noteworthy that acyclovir was not found to be effective in PR patients subjected to a randomized, triple-blind, placebo-controlled trial conducted in Uttar Pradesh, North India.

Only one PR sample (S4) was HBsAg positive but HBV DNA negative. This is not unusual as similar incidences have been previously reported in case of vitiligo patients with HBV infection. Most of the PR patients in the current study visiting a government hospital in Kolkata are from lower socioeconomic background and it is most likely that they have not been vaccinated against HBV or have undergone blood transfusion. It is most likely that these people contracted the disease either from infected mother during birth or breastfeeding or through horizontal transfer. A survey had been conducted in eastern parts of India which reported that intrafamilial childhood horizontal transmission played more important role in HBV transmission than sexual mode of transmission.

The HBV sequencing data suggest that samples S6, S9, S11, S12, and S13 have exactly the same sequence and samples S5, S8, and S10 have the exact same sequence in the partial S gene analyzed. These results raise the issue of possible cross-contamination across the samples that tested positive for HBV in this study. Nevertheless, all precautions had been taken to rule out PCR cross-contamination. One possibility is that S6, S9, S11, S12, and S13 contained the same virus; similarly, S5, S8, and S10 were infected by the same HBV. This is quite plausible given that the overall prevalence of OBI in this part of the globe has been reported to be unusually high by other workers. According to one study, out of 1027 HBsAg-negative donors, 188 were ant-HBc positive, and within them, 21% were HBV DNA positive. This implies that the frequency of OBI based on the above study was about 1 in 30 HBsAg-negative donors, against 1 in 1000–20,000 as observed globally. However, there are no reports so far on exactly why OBIs are increasing in India.
Perhaps, the above two HBV isolates are fixed in the population and silently circulating/spreading without showing the signs and symptoms of overt hepatitis but manifesting as PR. This is further strengthened by the observation that sample S7 shares similar amino acid backbone as S6, S9, S11, S12, and S13 in the S protein [Figure 2a] except for the SP→R change at 178 position. From this observation, it may be inferred that S7 is not a contamination from the aforesaid samples and that perhaps the HBV from these PR cases is continuously evolving by selecting for more occult HBV-specific/immune escape mutations to adapt efficiently to the host environment.

Conclusions

Globally, reports of the association between PR and HBV are sparse. This is the first report of possible association of PR with asymptomatic HBV infections. Our study also provides for the first time the molecular genetic evidence of association of Indian PR cases with HHV 7 (not HHV 6), often with HBV co-infection. One limitation of this study is that it had been carried out at a pilot scale, involving a small sample size. Still, our results emphasize on the importance of mass HBV vaccination and cautions against the routine use of steroids in PR management as this may pose the risk of virus reactivations resulting from immunosuppression.

Our findings from this pilot-scale study warrant a multicentric investigation involving larger sample size for PR cases and healthy controls to further validate the results.

Acknowledgments

The authors deeply acknowledge all the study participants for their willingness to be part of this study. SB acknowledges SERB, DST, Government of India, for an ECR grant. SR acknowledges UGC for his Junior Research Fellowship. The authors acknowledge CSIR-IICB for providing laboratory facilities for conducting the present work.

Financial support and sponsorship

The study was funded by the Department of Science and Technology, Government of India, in the form of an ECR grant (ECR/2016/000032) to SB.

Conflicts of interest

There are no conflicts of interest.

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