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REASSESSING THE CONTROVERSIAL ASSOCIATION: A BRADFORD HILL CRITERIA ANALYSIS OF HUMAN PAPILLOMAVIRUS IN LARYNGEAL CANCER

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Abstract

The role of human papillomavirus (HPV) in Laryngeal cancer (LC) has been extensively investigated globally, yielding conflicting results. Despite numerous attempts by different research groups to explore the potential association of HPV with LC using statistical metaanalysis, the findings remain controversial due to inherent limitations in meta-analytical approaches. Therefore, this study was conducted to investigate the potential link between HPV and LC using an alternative method, the Bradford Hill criteria, to provide a clearer perspective. Initially, we conducted a comprehensive search on PubMed to gather all studies associating HPV with LC. Subsequently, we examined the available data on HPV in LC and normal/benign samples, applying the major Bradford Hill criteria postulates to assess the potential association. Additionally, to enhance the reliability of our findings, we critically evaluated the methodologies of the identified studies to assess the risk of false-negative and false-positive results. Following a meticulous assessment of

previous studies against the Bradford Hill criteria postulates, it was observed that not all major postulates were fulfilled. Consequently, our findings suggest no causal association between HPV and LC.

Keywords: Laryngeal cancer (LC); Bradford Hill criteria; Human papillomavirus (HPV)

Introduction

Laryngeal cancer (LC) is one of the most common head and neck malignancies (1). At present, the increasing incidence of LC has threatened human life seriously. There are multiple factors of LC including drinking, smoking, air pollution, and sex hormonal imbalance. A recent pieces of evidence has suggested that human papillomavirus (HPV) infection is one of the main cause of LC (2).
Considering the participation of HPV in LC, worldwide different studies documented the role of HPV in LC so far, their results were contradictory (3-5). Various group of researchers used statistical meta-analysis to resolve this disagreement and obtain more accurate association between HPV and LC. However, due to significant limitations of the statistical meta-analysis including inability to critically evaluate the methodologies, providing no information regarding heterogeneity of the studied populations, and publication biasness, the evaluation of a correlation among HPV and LC is due with an additional strategy.

In our study, we evaluated the correlation among HPV and LC using Bradford Hill criteria postulates. These postulates are worldwide effective for linking a presumed cause with an effect [20]. In the evaluation, we analyzed the data of previous studies to document, whether or not previous studies met the Bradford Hill criteria postulates to declare a causal association among HPV and LC. Additionally, to make our outcomes more authentic, we also critically reviewed the methodologies of identified studies to address the propensity of false results.

Methods

In our study, we implemented a two-phase methodology (Figure 1).

Literature identification

Related studies associating HPV with LC were searched via PubMed using the keywords: “laryngeal cancer” AND “Human papillomavirus”. Additionally, “Retroviridae” AND “laryngeal intraepithelial neoplasia” were also used as a medical subject headings (MeSH) terms. Both, Mesh terms and keywords were combined in the search process. All the original articles were searched available till December 2020. In the end, we found a total of 754 original articles.

Relevant data acquisition

Out of 754 studies, in total relevant studies were shortlisted which studied the association between HPV and LC reading their titles, abstract, and the complete article. In addition, a detailed table was built after acquiring the required data from shortlisted studies.

Evaluation of the results using the postulates of Bradford Hill criteria

Based on the acquired data, we critically evaluated the selected studies using eight major Bradford Hill criteria postulates:
(1) Strength, (2) Temporality, (3) Consistency, (4) Plausibility, (5) Biological gradient, (6) Experiment, (7) Specificity, and (8) Analogy (9).

The postulate’s evaluation was descriptive, with no quantitative assigned score. The evidence for each postulate is given in (Table 1) and results part with a final verdict of whether or not the postulate was fulfilled.

Results

On PubMed, A total of 50 original studies (6-55) were identified worldwide that examined the potential link of HPV with LC. Table 1 summarizes the selected studies and includes the important acquired data from these studies essential for the assessment of Bradford Hill criteria postulates including information of the studied population, names of the technique utilized for the HPV identification, targeted gene name, name of the HPV detected strain, CI and P values, name of the prevalent identified HPV strain, total analyzed samples count (normal, benign and LC) with respective population-wide detection positivity ratios.

The positivity ratio of HPV detection in the LC samples was varied population-wide from 0% (24) to 100% (46). While, the positivity ratio of HPV detection in normal and adjacent/benign samples was varied from 0% (15, 24, 26, 36) to 5.6% (32) and 0% (12, 49) to 77% (22), respectively.

The evidence for each of the Bradford-Hill postulates

Strength

The existence of a weak association does not rule out the possibility of a causal association; however, weak this situation is more likely to be clarified by undetected prejudices. The point that stronger relationships tend to be more causative is rational. In total, 10 case-control studies (12, 15, 16, 22, 24, 26, 32, 36, 40, 49) were found in the literature reporting association between HPV and LC. Only (16, 32) of them have reported the CI (16), P-values (16, 32), and higher HPV detection ratio in LC samples as compared to the controls and found a significant association between HPV and LC in the population of China, Belgium, Turkey, Iran, Poland, USA and Japan except a single study (24) in Turkish populations, which did not reported a significant association. These data overall support a negligible strength of association between HPV and LC.

Consistency

Among (12, 15, 16, 24, 26, 32, 36, 40, 49) case-control studies, all the (12, 15, 16, 26, 32, 36, 40, 49) studies have reported the higher HPV detection ratios in LC samples relative to controls while a single study (22) has documented the opposite results and a study (24) has reported no HPV detection in both control and LC sample. Therefore, consistent findings have not been observed in different populations using different populations strengthening the existence of an actual effect.

Biological gradient

In certain circumstances, the effect can be the outcome of the minor existence of a factor while, in other cases, generally a greater exposures lead to the higher induction of an effect. Viral load measurements may predict whether HPV differential viral load leads to the differential outcomes in LC. Some studies (16, 22, 45) has reported the HPV viral load either in LC samples or benign sample. The study (22) showed the high HPV viral load in LC sample than benign sample while (16) showed similar viral load in control and LC samples. Therefore, biological gradient postulate was not fulfilled.

Temporality

Temporality refers to the necessity for HPV to precede LC. The HPV detection ratios scenario in the current study has shown different outcomes. In total, there was not a single cross-sectional study that has reported no HPV detection in LC samples, while a study (22) has reported the higher HPV detection ratio in benign relative to LC sample. There was no study which has reported the higher HPV detection ratio in normal controls relative to LC sample. Moreover, in few other case-control studies (16, 32, 36, 40) HPV was detected in both normal and LC samples. Such conflicting result thus, failed to fulfill the temporality postulate.

Plausibility

Plausibility refers to a proper mechanism between cause and effect. HPV is well recognized as a potent inhibitor of TP53 in cervical cancer by making aE6/E6AP/p53 complex, resulting in the degradation of TP53 protein (56). In literature, 4 studies (6, 19, 22, 29) found analyzing the association between HPV presence and expression variations in TP53 level: they have validated their results as TP53 was up-regulated (6, 22), down-regulated (29) and normally expressed (19) in the LC patients. Thus, the role of HPV in the etiology of LC is biologically not plausible.

Experiment

This postulate refers to the evidence from either animal or clinical studies. Evidence based on animal models and clinical studies, however, were absent in all the studies found in literature. Therefore, this postulate was not fulfilled.

Specificity

Causation is possible if a certain population develop LC in a certain region where the suspected cause is not clarified otherwise. Higher the specificity of the association between a factor and its effect, the more precise the relationship between a factor and its effect. LC is multi-factorial disease (57) and together with HPV the role of other non-infectious factors and oncogenic viruses (EBV and John Cunningham virus*)* in the development of LC is also well studied worldwide (58, 59). Thus, the complexity of the involved factors in LC development suggested no specificity.

Analogy

The similar diseases to LC that can considered to be LC analogous are breast cancer and cervical cancer caused by other viral agents like Epstein–Barr virus (EBV), and Mouse mammary tumor virus (MMTV) (60, 61). However, the role of MMTV and EBV in the development of breast cancer and cervical is yet not full established. Thus, in the present study, the scenario of analogy also suggests no association between HPV and LC.

Discussion

LC has the second highest incidence of head and neck malignant tumors worldwide (2). So far, many studies were conducted worldwide documenting the relationship between HPV and LC to identify the possible oncogenic pathways regulating by HPV in the development of LC, however the findings were inconsistent. In addition, a statistical meta-analysis has also been performed by different groups of scientist worldwide to generate a more meaningful relationship between HPV and LC, due to statistical meta-analysis shortcoming, scientists yet again failed to find a reliable relationship among HPV and LC. Therefore, in the present study our aim is to find a relationship between HPV and LC using Bradford Hill criteria postulates.

In total 50 original articles (6-55) were included in the present study. The HPV detection ratio reported in these studies was varied between 0% (24) to 100% (46) in LC samples. In most of the case-control studies (12, 15, 16, 24, 26, 32, 36, 40, 49) the positivity ratio of HPV detection was higher in the LC samples as compared to the controls while in a single study (22) HPV positivity ratio was higher in the controls in comparison to the LC samples.

Best to our knowledge, no study has applied the Bradford Hill postulates so far to identify the association between HPV and LC, However, one study utilized these postulates to analyze the causal association between Zika infection and microcephaly, and they suggested no link between the studied parameters (62).

Since, from the initial identification of HPV in LC, more evidence has become available. We systematically applied Bradford Hill's postulates on the available evidence to find an association between HPV and LC. The results were not in favor of a casual association. Therefore, we speculated that HPV along with other different viruses like human immunodeficiency virus (HIV)

and hepatitis and C virus (HCV and B), as well as other genetic abnormalities, smoking, alcohol consumption increases the risk of developing LC by affecting the body's immune system (63).
Moreover, deficiencies as well as and some of the major drawbacks linked with the methodologies of the included studies have been discussed below.

Possible causes of false-negative results
Few studies did not detected HPV in any of the LC or control samples they were utilizing. How we can be sure that the negative results were not because of the low-quality DNA? Several studies used positive control to address the question (6, 7, 12-14, 19, 20, 22, 24, 29-31, 36, 37, 39-43, 51, 53-55) however, other studies (8-11, 15-18, 21, 23, 25-28, 32-35, 38, 44-50, 52) did not utilized the positive control in their experiment, there is no way to validate their negative findings.
Primers selection targeting L1 and E1 genes of HPV might be inefficient for detecting HPV presence in the advanced carcinoma and thus results in false negative, since L1 and E1 regions might be lost during viral genome integration with the genome of host, whereas, the E6/E7 regions remained consistently present in any circumstances so, this is the plausible explanation for the completely negative results of (12, 26, 36).

Possible causes of the false-positive results
Most of the summarized studies utilized PCR (7-12, 14, 15, 18, 21, 23, 25, 26, 28, 32, 34-36, 38-41, 43, 46, 50, 54, 55) for the HPV detection but only 23 studies (6, 13, 16-19, 22, 27, 29, 30, 33, 45, 47, 48, 51-53) used a second technique to validate their PCR results including southern blot (6, 53), *In-situ* hybridization (13, 17, 24, 33, 42, 47, 48, 51), immunohistochemistry (16, 20, 22, 27, 29-31, 37, 39, 42, 45, 48, 52), reverse dot blot method (19) and their results shows a deviation from the PCR results except in one study (51) which exhibited similar results to the PCR.

Expression profiling of different genes including p14, p16, p53, RB, and some other genes could be used as a surrogate biomarker in HPV positive LC patients. In addition to the HPV detection, expression profiling of these biomarkers were also done by many studies (6, 7, 13-17, 19, 20, 22, 23, 27, 29-31, 33, 37, 39, 41, 42, 44, 45, 48, 49, 52-54), to further validate their findings, out of which 25 studies (6, 7, 13-17, 19, 20, 22, 23, 27, 29-31, 33, 37, 39, 42, 44, 45, 48, 49, 52-54) have validated their findings by analyzing p53, RB and other surrogate biomarkers while the other 2 studies (20, 41) were failed to validate their findings with respect to surrogate biomarkers. Such inconsistencies in the previous studies results pose a significant question mark as to the choice of suitable methods and their sensitivity.

Comparison of normal, benign and malignant samples
Case-control studies are essential when looking for a causal association between cause and the disease. Few of the selected studies analyzed the LC samples only (6-11, 13, 14, 17-21, 23, 25, 27-31, 33-35, 37-39, 41-48, 50-55) which did not allow a comparison with normal or adjacent / benign and LC samples. However, on the other side, few of the selected studies (12, 15, 16, 22, 24, 26, 32, 36, 40, 49) analyzed both normal or adjacent/benign and LC samples, and this comparison revealed a higher HPV detection ratio in LC samples in (12, 15, 16, 26, 32, 36, 40, 49) studies while lower in other studies (22) as compared to the control. However, no study has found a correlation between HPV and a certain LC subtype or histologic grade.
The findings of this study have reported no causal association between HPV and LC. However, considering the constrains in the methodologies of the previous studies, additional experiments are proposed to prove a proper HPV etiology in LC.

Acknowledgements
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Conflict of interest
None to declare

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Figures legend

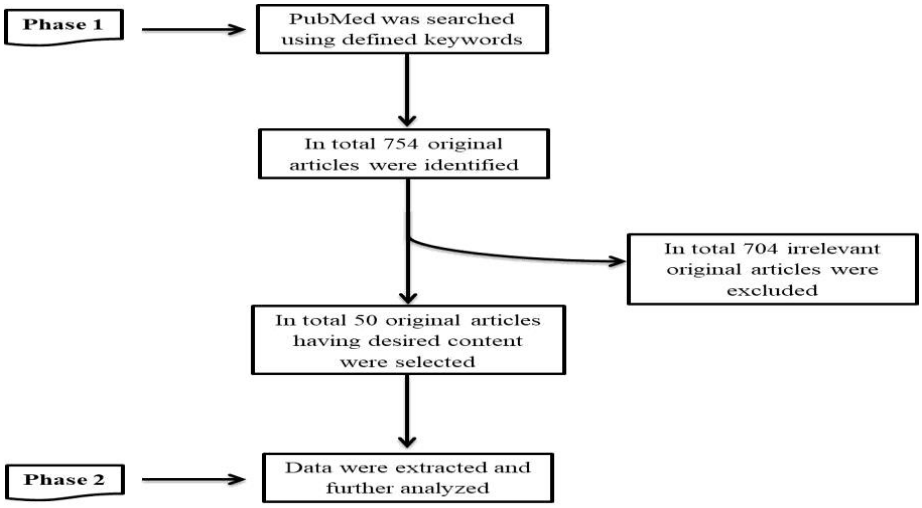

Figure 1: Overview of the methodology implemented in the present study.

Table 1: Summary of the detection of HPV and positivity rate in normal and vulvar cancer samples relative to the different selected articles.

Studied Population	Technique used for viral genome detection	Target gene/protein	Prevalent strain	Number of normal samples screened (control)	Percentage positivity of HPV in normal samples (%)	Number of the adjacent or benign samples screen	Percentage positivity of HPV in adjacent or benign samples (%)	Number of the total laryngeal cancer samples screened	Percentage positivity of HPV in laryngeal cancer samples (%)	References	p-value	CI
	PCR, Southern blot	E6/E7	16, 18, 33, 6, 11	0	0	0	0	102	58.8	(6)		
	PCR	L1	16, 18, 31, 33, 45	0	0	0	0	674	4.9%	(7)		
	PCR	E2, E6	16	0	0	0	0	86	2.4%	(8)		
	PCR	E6, E7	16	0	0	0	0	50	58%	(9)		
	PCR	E6, E7	16	0	0	0	0	31	19.4%	(10)		
	PCR	L1	16	0	0	0	0	163	1.8%	(11)		
	Qrt PCR	L1, E7, E6	16,18	0	0	19	0	84	27.4	(12)		
	in situ hybridization, PCR		16	0	0	0	0	211	62.6%	(13)		
	p16INK4A immunohistochemistry	L1	16, 18, 45, 53	0	0	0	0	318	10.1%	(14)		
	PCR	-	1, 6, 8, 11, 13, 16, 18, 30, 31, 32, 33, 45, 51	6	0	0	0	93	36	(15)		
	PCR & P16 immunohistochemistry	E2 and E6	16	300	2.7%	0	0	300	6.7	(16)	0.28	1.21–6.68
	PCR, in situ hybridization	L1, E6, E7	16,18	0	0	0	0	52	32.7	(17)		
	PCR	L1	16,18	0	0	0	0	84	34.5	(18)		
China	PCR-RDB	E7	16	0	0	0	0	332	13.55	(19)		
Tunisia	in situ hybridization	E6	No relevance found	0	0	0	0	70	55.71	(20)		
Spain	PCR	E6, E7	16, 31, 33, 35, 43, 53, 58,61, 62, 66, and 70	0	0	0	0	123	22.76	(21)		
Belgium	PCR	E6, E7, L1	16	0	0	39	77	67	75	(22)		
Turkey	PCR	-	16 and 18	0	0	0	0	90	12.2%\	(23)		
	In-situ hybridization	E6	-	11	0% (all negative)	0	0	82	0% (all negative)	(24)		
	PCR		6, 11,42, 43, 44, 16, 18, 31, 33,35, 45, 51, 52, 56	0	0	0	0	50	14	(25)		
	PCR	L1	6,11,16	5	0	0	0	95	7.36	(26)		
	Rt PCR	E6	16	0	0	0	0	78	26.02	(27)		
Lithuania	PCR, qRT-PCR	E1, E2	16, 18	0	0	0	0	49	42.86	(28)		
	Immunohistochemistry	E6	6,11,16,18,31,35,33,39,45,56,58,67,68	0	0	0	0	1042	5.7	(29)		
	PCR & P16 immunohistochemistry	E6	HPV16	0	0	0	0	62	3.22	(30)	0.023	
Australia	Immunohistochemistry	E6, E7	HPV16	0	0	0	0	307	6.5%	(31)		
Iran	PCR	L1, E6	16	36	5.6	0	0	44	25	(32)	0.235	
Austria	Immunohistochemistry	-	HPV16	0	0	0	0	85	11%	(33)		
Poland	PCR	-	HPV single infection	0	0	0	0	18	33.33%	(34)		
	PCR	L1	16	0	0	0	0	50	36	(35)		

	Pcr/DEIA	L1	16,33,18	22	0	49	8.2	93	35.5	(36)		
United states	PCR & immunohistochemistry	E6/7	-	0	0	0	0	49	14.3	(37)		
	PCR	L1	16, 18, 31, 33,35, 39, 45, 51, 52, 56, 58, 59, 66, and 68	0	0	0	0	148	21	(38)		
	PCR	L1	16, 26, 31, 39, and 52	0	0	0	0	38	16	(39)		
	PCR	L1	16,18	0	0	22	27	18	78	(40)		
	PCR	E6	16	0	0	0	0	79	27	(41)		
	In situ hybridization	E6, E7	16	0	0	0	0	73	2.7	(42)		
	PCR	E6, E7, L1	-	0	0	0	0	83	42.3%	(43)	0.788	0.866
	In situ hybridization, immunohistochemistry	-	0	0	0	0	0	38	11%	(44)		
Germany	PCR	E6, L1	16,56,45,53,70,11,42,33	0	0	0	0	92	35	(45)		
	PCR	E6, E7	6, 11	0	0	0	0	44	100	(46)		
Taiwan	PCR & in situ hybridization	-	16	0	0	0	0	106	13.2	(47)		
	In situ hybridization	L1	6,18,11,16,68	0	0	0	0	89	45.6	(48)		
Japan	Hybrid Capture 2 assay method	-	16, 18, 31,33, 35, 39, 45, 51, 52, , 11, 42,43, 56, 58, 59 and 68	0	0	15	0	12	25	(49)		
	PCR	-	6, 11, 16, 18, 31, 33, 35, 52b and 58	0	0	0	0	34	41.2	(50)	0.042	
Greece	NPCR and in situ hybridization	L1, E6. E7	6,16,18,33	0	0	0	0	154	13	(51)		
Italy	PCR	E6	HPV16	0	0	0	0	94	1	(52)		
	PCR	E6, E7	16,18,33	0	0	0	0	75	29.3	(53)		
Mexico	PCR	E6, L1	HPV 16	0	0	0	0	30	20	(54)		
Denmark	PCR	L1, E1	-	0	0	0	0	30	3	(55)		

PCR = Polymerase chain reaction